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TITLE: Prevention of Ovarian High-Grade Serous Carcinoma by Elucidating Its Early Change

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14. ABSTRACT: : Project 1 We will determine the early molecular changes in STIC and their biological significance in developing high-grade serous carcinoma. marker selection and sample preparation will begin in the next coming months. Project 2 We will evaluate whether the presence of a STIC is associated with different clinical manifestations and/or outcome compare to those patients in whom a STIC was not identified. Molecular profiling will be initiated after quality control checking. Project 3 We will identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an <i>in vitro</i> system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma. Project 4 We plan to if the statin drugs are effective in preventing STIC formation and suppress tumor progression in the OVGP1 mouse model that spontaneously develops STIC and neoplasms.. Project 5 With the data and cases piling up, we will be able to address the molecular and epidemiologic profile of putative precursor lesions including STIC in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. Also, a pilot study will be performed to determine the most cost-effective way to prepare the tissue sections for studies related to study early tumor development in ovarian cancer. This information will be shared with science community.					
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Section I- Purpose and Scope of the Research Effort (all projects)

The purpose of this Ovarian Cancer Consortium is to test the overarching hypothesis that serous tubal intraepithelial carcinoma (STIC) is the precursor and not a metastasis of many, if not most, pelvic high-grade serous carcinomas (HGSCs) but we believe all the other proposed candidates should be investigated in order to determine if STIC is the precursor of all ovarian and pelvic HGSCs or that OSE and CICs harbor precursor lesions as well. Our objective is to then carefully characterize the morphologic, molecular genetic, immunohistochemical (IHC) and epidemiologic features of the precursor lesions(s) (Projects 1-5). If STIC is shown to be the precursor lesion, the data generated by our studies will provide the rationale for our long-term objective, which is the prevention of ovarian HGSC by surgical or medical approaches. Opportunities in the field of cancer prevention have never been greater and therefore our Consortium will undertake innovative studies aimed at providing the scientific underpinning for reducing the burden of ovarian cancer through prevention. Finally, it is important to note that clear cell, endometrioid and mucinous carcinomas are clinically important but they represent only 25 % of all ovarian carcinomas and account for 10% of deaths. In contrast, as noted above, HGSC represents 75% of all ovarian cancers and accounts for 90% of the deaths. Accordingly, we will focus our studies exclusively on the early events associated with HGSC, as it clearly is the most important histologic subtype in terms of frequency and mortality.

The main research efforts in this Consortium are summarized in our five projects.

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.

Project 2: Evaluate all the proposed site of origin (FTE, OSE, CICs and peritoneum) showing that the morphologic and molecular features of tubal, ovarian and primary peritoneal HGSCs are the same and in conjunction with Project 1 confirming our hypothesis that many, if not most, HGSCs originate in the fimbria and involve the ovary secondarily.

Project 3: Identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an in vitro system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma.

Project 4: Locate and characterize precursor lesions of “ovarian” cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in “ovarian” carcinogenesis using human tubal xenografts in nude mice.

Project 5: Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents as OCPs in particular are known to prevent ovarian cancer and impact survival.

Section II, III and IV (5 projects are reported here individually)

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the

ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.

Research site: Johns Hopkins University

Project Leader: Ie-Ming Shih

Co-investigators: Doug Levine (MSKCC), Robert J. Kurman (JHU)

Section II. Progress to Date

Task 1. Determine the clonal relationship and tumor progression pathway from STIC to invasive high-grade serous carcinoma.

Task 1a. Case selection and sample preparation including LCM, DNA extraction (1-20 months)

Progress: Project 1 is requesting the new STIC cases from the Pathology Core to perform this study. Previously we have collected STIC cases studies (1-4), and now we are collecting new cases from different research sites. The actual number of STICs and associated HGSCs will be known and reported after Pathology Core has compiled all the cases and after centralized pathology review.

Task 1b. TP53 mutational analysis of all the potential precursor lesions of HGSC (8-24 months).

Progress: The task has been performed and the results have been described in the last progress report and, most importantly, the data have been recently published in J Pathol, 226:421-426, 2012. PMID:21990067. So we will not reiterate the progress here.

Task 1c. Allelic imbalance assay by digital SNP analysis and data analysis (24-36 months)

Progress: Not yet performed.

Task 2. Determine the early molecular changes associated with serous tubal intraepithelial lesions.

Task 2a. Immunohistochemistry study on ovarian cancer-associated markers on STICs and other putative precursor lesions (18-40 months)

Progress: We have finished a very interesting study by analyzing ALDH1A1 in normal fallopian tube, STIC and HGSC. Below, we first summarize the findings then give the details regarding the background and rationale to do this study as well as the main findings and impact on this research field.

Summary of this new study: Tumor-initiating cells are thought to share features with normal somatic stem cells. In mice, stem cells at the ovarian hilum have been shown to express the stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1A1), and are prone to malignant transformation. However, whether these findings are relevant to humans has not been investigated. In this study, we applied immunohistochemistry to assess the distribution of ALDH1A1 expression in epithelium from human fallopian tubes, with particular focus on sites of transition from tubal epithelium to mesothelium (i.e. tubal-peritoneal junctions) and serous tubal intraepithelial carcinoma (STIC), the putative precursor lesions of ovarian high grade serous carcinoma (HGSC) as well as in HGSC. ALDH1A1 staining was detected in 20%-80% of epithelial cells from normal fallopian tube and from the tubal-peritoneal junctions. In contrast, ALDH1A1 immunoreactivity was undetectable in the majority of STICs (15/17, 88%) and all “p53 signatures” (11/11, 100%). In ovarian HGSC, ALDH1A1 positive cancer cells were generally very few, and there is no difference in ALDH1A1 expression levels between matched primary and recurrent tumors. *In-silico* analyses of the mRNA expression dataset from The Cancer Genome Atlas demonstrated downregulation of ALDH1A1 in ovarian HGSC as compared to normal fallopian tube, and no significant association between ALDH1A1 expression levels and overall survival. Our results do not support ALDH1A1 as a marker of stem cells in human fallopian tube and demonstrate that loss of its expression, indeed, is an early event in the development of HGSC.

Background and Rationale: There is increasing recognition that a significant proportion of pelvic high grade serous carcinomas (HGSC) arise from a non-invasive microscopic lesion in the distal fallopian tube, known as serous tubal intraepithelial carcinoma (STIC). These foci are characterized by marked cytologic atypia and are thought to be the immediate precursors of HGSC, having been identified in prophylactic salpingo-

oophorectomy specimens from women with germline BRCA mutations, as well in the tubal fimbriae adjacent to sporadic ovarian HGSC. Thorough examination of fallopian tubes has revealed putative premalignant lesions, which may precede the development of STIC. These include focal proliferations of morphologically normal secretory cells marked by diffuse p53 immunoreactivity, termed “p53 signatures,” which may represent early clonal expansions of tumor-initiating cells. Experimental work conducted in a variety of model systems across different tumor types have consistently demonstrated that tumor-initiating cells are derived from either a somatic stem cell or an early progenitor cell that has reacquired stem cell features. This is in keeping with the concept that the capacity for indefinite proliferation, or self-renewal, is essential for malignant transformation⁷. Studies have also shown that only a fraction of cancer cells, known as “cancer stem cells” are capable of forming tumors upon serial transplantation into immunocompromised mice, a technique used to assess self-renewal⁸. One of the most commonly used markers for both normal and malignant stem cells is aldehyde dehydrogenase isoform 1A1 (ALDH1A1, previously known as ALDH1). ALDH1A1 is an enzyme involved in the production of retinoic acid, which has been implicated in cellular differentiation⁹. Overexpression of ALDH1A1 has been associated with poor prognosis in several malignancies, including carcinomas of the breast and pancreas. In mice, the junction between the ovarian surface epithelium and tubal epithelium is enriched for ALDH1A1-positive stem cells that show increased susceptibility to malignant transformation. The analogous structure in humans, the tubal-peritoneal junction, has been postulated to be a hotspot for carcinogenesis; however, it is unclear whether these regions are enriched for stem cells, as seen in the mouse model. To gain further insight into the phenotype of the cell of origin and the early events in the pathogenesis of HGSC, we applied immunohistochemistry to determine the pattern of ALDH1A1 expression in fallopian tubes including the tubal-peritoneal junctions, STIC and HGSC.

Results: To determine the expression of ALDH1A1 protein expression on tissue sections, we used two independent antibodies from Epitomics and BD. Quality control measures were taken to assess the sensitivity and specificity of the ALDH1A1 antibodies. For both

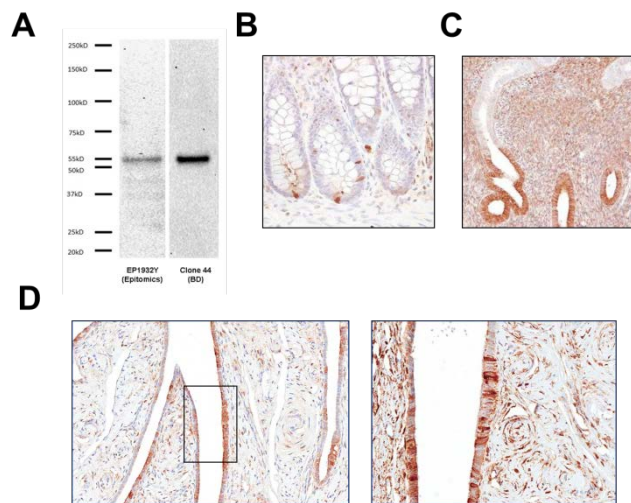


Fig. 1. ALDH1A1 expression in HHL-6 cells and human tissues. A. Western blot analysis using both Epitomics and BD antibodies shows a robust band (~55 kD) corresponding to the ALDH1A1 protein on HHL-6 human hepatocyte cell lysate. B. Discrete staining of single cells at colon crypt bases. C. Strong staining of deep portions of endometrium stratum basalis. D. In normal fallopian tube epithelium, ALDH1A1 is expressed in tubal epithelial cells.

antibodies, western blot performed on protein extracts from immortalized human hepatocytes revealed a robust 55 kD band, corresponding to the molecular weight of ALDH1A1 (Fig. 1A). The result suggests the specificity of both antibodies in detecting ALDH1A1 in immunohistochemistry. Using both Epitomics and BD antibodies, we found ALDH1A1 positive cells restricted to discrete single cells at the colonic crypt bases, where intestinal stem cells are known to reside (Fig. 1B). In endometrial glands, more diffuse immunoreactivity was observed, though localized to the deep portions of glands in the stratum basalis (Fig. 1C). Endometrial stromal cells were also positive for ALDH1A1 staining. Patterns of staining in those tissues were comparable between the two ALDH1A1 antibodies, with a slightly higher background staining observed with the BD antibody. Therefore, we mainly used the Epitomics antibody in determining ALDH1A1 staining pattern in this study.

In normal tubal epithelial cells, ALDH1A1 staining was detected in all 25 cases. The staining pattern was always patchy and discontinuous, and in some areas, strong staining marked discrete single cells, both secretory and ciliated (Fig. 1D); however more often, long stretches of epithelium showed diffuse immunoreactivity. There were no clear differences in the percentage of ALDH1A1-positive cells observed in sections of fimbriae compared with more proximal regions of the tube ($p > 0.05$). As in endometrium, prominent staining was observed in stromal cells.

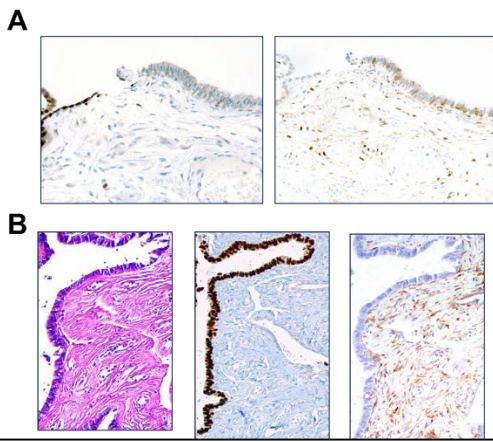


Fig. 2. ALDH1A1 staining in the tubal-mesothelial junctions and in a p53 signature. A. A tubal-mesothelial junction. The mesothelium is diffusely positive for calretinin while tubal epithelium is negative. ALDH1A1-positive cells are present in the fallopian tube close to the junction. B. A p53 signature which shows normal morphology of tubal epithelium but is stained with p53. The p53 signature epithelial cells are completely negative for ALDH1A1 immunoreactivity.

Recent work based on a mouse model has identified ALDH1A1-positive stem cells enriched at the ovarian hilum¹², an anatomical structure which we presumed to be analogous to the tubal-peritoneal junctions in human fallopian tube. Tubal-mesothelial junctions from 28 specimens were identified by morphology, with uncertain cases confirmed by calretinin immunostaining. We found ALDH1A1-positive epithelial cells in 15 (54%) of 28 junctions and a representative case was shown in Fig. 2A. The percentage of ALDH1A1-positive cells in those junctions was similar to that in normal tubal epithelium elsewhere ($p > 0.05$). In this set of normal fallopian tubes, there were a total of 11 p53 signatures of which normal-appearing tubal epithelial cells show intense p53 staining. Interestingly, the p53 signatures did not show ALDH1A1 immunoreactivity (Fig. 2B).

Next, we assessed ALDH1A1 expression in STICs and HGSCs. ALDH1A1 staining was detected in 0 of 16 STICs and in 41 (61%) of 67 HGSC. Among 16 STIC cases, there were 11 containing HGSC on the same sections that allowed to direct compare the staining pattern between STIC and HGSC from the same patients. We found that the staining patterns were generally consistent between STIC and HGSC components from the same cases (Fig. 3A), with the exception of two specimens, in which focal positive staining was detected in HGSC but not STIC. Although many HGSCs expressed ALDH1A1, it should be noted that most of those positive tumors had a very low percentage of ALDH1A1-positive cells. For example, among 41 ALDH1A1-positive cases, about half (19 cases) had percentages greater than 5%. In HGSCs, there were 26 paired primary and recurrent HGSCs and we did not observe significant difference in the percentage of ALDH1A1-positive cells between primary and recurrent groups. Matched primary and recurrent HGSC had comparable frequencies of ALDH1A1-positive tumour cells (mean \pm SD: $8.0 \pm 14.6\%$ versus $4.5 \pm 7.7\%$, $p = 0.28$). The percentage of ALDH1A1-positive cells in different groups of specimens was summarized in Fig. 3B. There was a significant decrease in the percentage of ALDH1A1-stained cells in STIC and p53 signatures as compared to normal fallopian tubes including the tubal-mesothelial junctions ($p < 0.0001$).

To confirm our results based on immunohistochemistry, we undertook several approaches. First, we stained representative cases using the BD ALDH1A1 antibody (clone 44). The

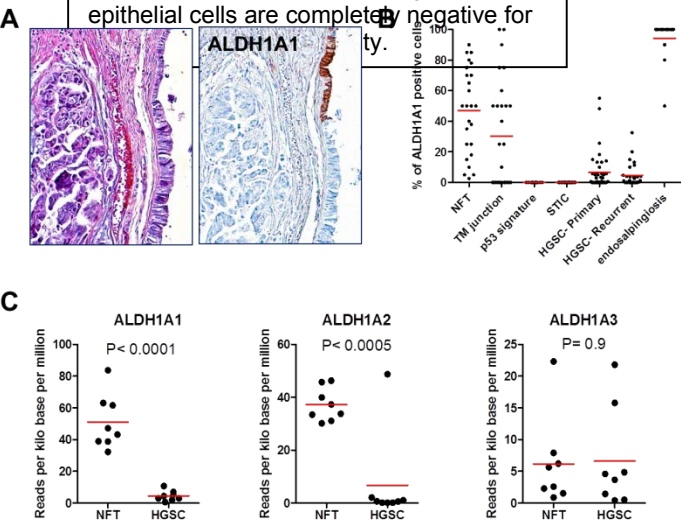


Fig. 3. Expression of ALDH1A1 in normal fallopian tubes and different types of lesions. A. A STIC shows complete absence of ALDH1A1 staining while the adjacent normal tubal epithelium is intensely positive. B. The scatter plot shows percentage of ALDH1A1-positive cells in individual specimens. The red lines are the means from each tissue group. C. RNA sequencing demonstrates a significantly higher level of ALDH1A1 and ALDH1A2 mRNA in normal fallopian tube (NFT) than high-grade serous carcinoma (HGSC). The red lines are the means.

performance of both antibodies was comparable, with no appreciable differences in the distribution of staining and the percentage of ALDH1A1 positive cells. Second, based on RNA sequencing (Sherman-Baust et al. unpublished), we compared the RNA expression levels (based on reads per kilo base per million) of ALDH1 in 8 samples of fallopian tubes of which the epithelial cells were directly prepared for RNA sequencing and 8 HGSCs. We found a highly significant decrease in ALDH1A1 in HGSCs as compared to normal fallopian tube epithelium (Fig. 3C). In this study, other ALDH1 related genes, ALDH1A2 and ALDH1A3, were also analyzed. ALDH1A2 also showed a similar pattern to ALDH1A1 but there was no difference in ALDH1A3. Lastly, in-silico analysis of TCGA mRNA expression data for ovarian HGSC19 revealed decreased expression in HGSC relative to normal fallopian tube (tumour/normal expression ratio = 0.13, $p < 0.0001$). ALDH1A1 expression levels in primary ovarian HGSC were not associated with overall survival (HR = 1.04, $p = 0.757$) (Fig. 4).

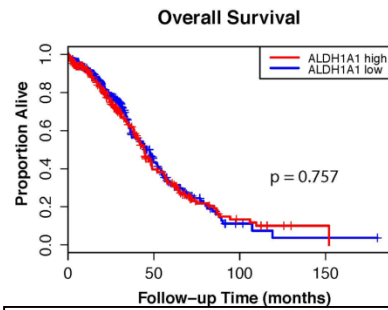


Fig. 5. K-M analysis shows no difference in the overall survival in HGSC patients whose tumors express high or low levels of ALDH1A1. This is based on analysis of the TCGA database.

In addition, we have also collaborated with Project 4 in studying the expression of topoisomerase II in ovarian cancer tissues and STIC. Please see the attached manuscript in Project 4 Report for details.

Task 2b. In situ hybridization and/or mRNA expression analysis on those markers that the antibodies are not available (30-40 months)

Progress: So far, we have identified good antibodies (laminin C1, ALDH1A1, p53, topoisomerase II, cyclin E1, etc.) for immunostaining purposes and will consider in situ hybridization if we can't find the appropriate and specific antibodies.

Task 2c. Verification of new markers from Project3 in precursor lesions (24-56 months)

Progress: This task has not yet started as we are waiting for new markers generated from other Projects.

Task 2d. Telomere FISH on STICs and other precursor lesions (36-48 months)

Progress: Part of the task has been performed and the results have been published before the consortium grant started. Since we have established the method, we will be able to perform telomere FISH on new STICs and other precursor lesions as proposed once we have the specimens ready for study.

Task 2e. Data analysis and preparation for publications (40-60 months)

Progress: We are in the process of generating data although we continue publishing our results.

Section III. Problem Areas for Project 1

As discussed in the previous progress report, the problem area is mainly on Task 1C. This is because the technology as proposed is likely labor intensive and, more importantly, costly, we are seeking a more cost effective technology to count alleles. Since that the methods of allelic counting have advanced significantly in recent years, we are evaluating the currently available technical platforms for the study in this task. After our preliminary study, we feel that digital PCR platform is arguably the most appropriate and cost-effective platform but it requires purchase of the equipment. There are two platforms in the market including OpenArray® Real-Time PCR Platform from Life Technologies and RainDrop™ Digital PCR System from Fisher Scientific. However, we do not have the budget to purchase the equipment and hoping that the core facility would have one available for us to use in the near future. Another problem area is that we propose to focus on BRCA1 immunohistochemistry in STIC and associated high-grade serous carcinoma. Although the antibody condition and method have been established by investigators from Project 2, the sensitivity and specificity of this antibody to detect the intact BRCA1 proteins have remained a concern from the molecular perspective. Several research groups including our own are looking into this problem and we will consider performing the BRCA1 immunostaining when we have a clearer picture about the natures of the antibody.

Section IV. Future Works in Project 1

Task 2a. Immunohistochemistry study on ovarian cancer-associated markers on STICs and other putative precursor lesions (18-40 months)

We will wrap up the study as presented above and submit the manuscript for publication. Other markers in line to be considered for study include M30 (an apoptotic marker), topoisomerase II, and new markers identified from the Project II.

Task 2b. In situ hybridization and/or mRNA expression analysis on those markers that the antibodies are not available (30-40 months)

We are currently applying FISH analysis of *CCNE1* which represents the most frequently amplified gene in high-grade serous carcinoma. This is an ongoing project and hopefully we will summarize the new findings in the next progress report.

Task 2d. Telomere FISH on STICs and other precursor lesions (36-48 months)

As discussed in the last progress report, we have recently identified somatic mutations in the telomerase promoter in certain types of human solid tumors. The promoter mutations are related to increase telomerase activity and subsequently an increase in telomere length. Now, we are sequencing a cohort of high-grade serous carcinomas first and if we identify the mutation rate > 10%, we will perform mutational analysis in STIC specimens. It is expected that we will report this finding in the next progress report.

References cited for Project 1 report:

1. Kuhn E, Kurman RJ, Sehdev AS, et al. Ki-67 labeling index as an adjunct in the diagnosis of serous tubal intraepithelial carcinoma. *Int J Gyn Pathol*. 2012;in press.
2. Kuhn E, Kurman RJ, Soslow RA, et al. The Diagnostic and Biological Implications of Laminin Expression in Serous Tubal Intraepithelial Carcinoma. *The American journal of surgical pathology*. 2012.
3. Kuhn E, Kurman RJ, Vang R, et al. TP53 mutations in serous tubal intraepithelial carcinoma and concurrent pelvic high-grade serous carcinoma- evidence supporting the clonal relationship of the two lesions. *The Journal of pathology*. 2012;226:421-426.
4. Kuhn E, Meeker A, Wang TL, et al. Shortened telomeres in serous tubal intraepithelial carcinoma: an early event in ovarian high-grade serous carcinogenesis. *Am J Surg Pathol*. 2010;34:829-836.

Project 2: Evaluate all the proposed site of origin (FTE, OSE, CICs and peritoneum) showing that the morphologic and molecular features of tubal, ovarian and primary peritoneal HGSCs are the same and in conjunction with Project 1 confirming our hypothesis that many, if not most, HGSCs originate in the fimbria and involve the ovary secondarily.

Research site: Memorial Sloan-Kettering Cancer Center

Project Leader: Douglas Levine

Section II – Progress to date

Project 2: Evaluate all the proposed site of origin (FTE, OSE, CICs and peritoneum) showing that the morphologic and molecular features of tubal, ovarian and primary peritoneal HGSCs are the same and in conjunction with Project 1 confirming our hypothesis that many, if not most, HGSCs originate in the fimbria and involve the ovary secondarily.

Task 1: Determine the frequency of STICs in patients with advanced pelvic HGSC.

Task 1a. IRB documents filed at each consortium sites. (Complete)

IRB documents have been completed by each site in the consortium.

Task 1b. Case identification. (Complete)

We have identified 228 cases of HGSC from MSKCC since 2009 when the SEE-FIM protocol was initiated. We have developed a data dictionary and data collection form for other consortium centers to contribute clinical data regarding STIC cases. Each center has agreed to provide additional clinical data to develop a more robust dataset.

Task 1c. Slides review and section recut (Complete).

Of the 228 cases identified from institutional databases, 18 cases were unavailable and 33 cases had insufficient tissue sections available to determine that a STIC lesion could not have been present. Of the remaining 177 cases, 104 are confirmed to have STIC lesions and 73 do not have STIC lesions based on comprehensive review of fully submitted fallopian tubes.

Task 1d. Tabulate frequency of STIC (Complete).

Progress: The frequency of fallopian tube cancer in this population is 59% and is much higher than early historic populations supporting the increase in diagnosis of tubal carcinoma with the advent of comprehensive tubal analysis.

Task 2: Evaluate whether the presence of a STIC is associated with different clinical manifestations and /or outcome compare to those patients in whom a STIC was not identified.

Task 2a: Collect clinical data from specimens identified in Task 1a, above. (Complete)

Clinical data has been collected from all MSKCC patients. Of the 215 patients identified, the median age was 62 years old; identical to population based reports of HGSC confirming the fact that our study population reflects the general population of HGSC patients. All patients had high-grade (grade 3) serous carcinoma with stage IIIC (N=159, 74%) or IV (N=56, 26%) disease. Optimal surgical cytoreduction was achieved in 194 patients (90.2%). All patients received platinum-based adjuvant chemotherapy after surgery. With relatively short median follow-up of 23 months, 12% of the patients have died and the median overall survival has not yet been reached.

Task 2b: Analyze clinical variables in the context of specimens with or without STIC

The presence or absences of STIC lesions has been confirmed in all cases from MSKCC that will qualify (sufficient tubal representation in pathology specimens). We will now be able to finalize our analysis of the clinical data in the context of STIC lesions for MSK cases. Additional analyses are ongoing based on further collection of cases from other consortium sites.

Task 2c: Write manuscript and publish results

Pending. We will await data from other consortium institutions and then incorporate these findings into Task 2b.

Task 3: Compare the molecular features of advanced pelvic HGSCs with and without associated STIC to various normal pelvic tissues.

Task 3a. Collect 100 HGSC specimens that had SEE-FIM processing from Consortium sites; 10-20 specimens per site with the balance contributed from MSKCC

We have identified 153 advanced ovarian cases across the consortium and have extracted nucleic acids from 102 cases with and without STIC lesions that yielded high quality nucleic acids. (50 STIC cases and 52 non-STIC cases, See Fig 1). We have received 29 qualifying HGSC cases from other consortium centers that have had comprehensive evaluation of the fallopian tubes as shown in Table 1. We were not able to receive any specimens from Yale as they do not have access to any retrospectively banked specimens. We have discussed the design issues again with Dr. Cope (Biostatistics core) and will run tumors and normal samples each as a single but separate batch.

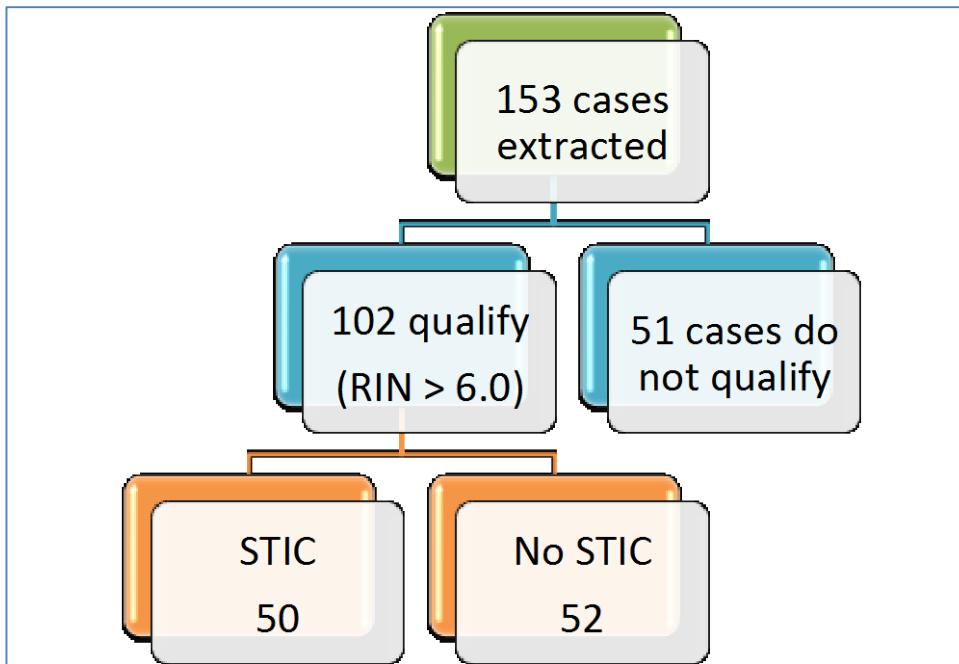


Figure 1. Consort diagram of case qualification for Task 3a.

	STIC	No-STIC	Total
MSKCC	44	29	73
Hopkins	3	10	13
Toronto	5	11	16
All institutions	52	50	102

Task 3b. Collect 10 normal tissues from each of 5 anatomic sites; 50 total tissues. Tissues to be collected across Consortium sites, with each site contributing at least 2 of each normal tissue type with the balance contributed from MSKCC

To begin to address this task, we have optimized the methods for collecting normal tissue (SOP appended below). We have confirmed by cytology that these samples contain a predominance of epithelial or mesothelial cells, as appropriate. We are also able to recover 50-300 ng of DNA or RNA per sample. Approximately 50%

of collected specimens yield good quality RNA (Fig 2.). We have collaborated with Dr. Tanner from Hopkins to collect normal samples. A MSK lab member travelled to Hopkins to train Dr. Tanner in collection technique. We have received one sample to date that failed QC. Dr. Shaw from Toronto has indicated that she will begin to collect normal brushings as outlined in the SOP. Progress continues on this task. No new data for this report.

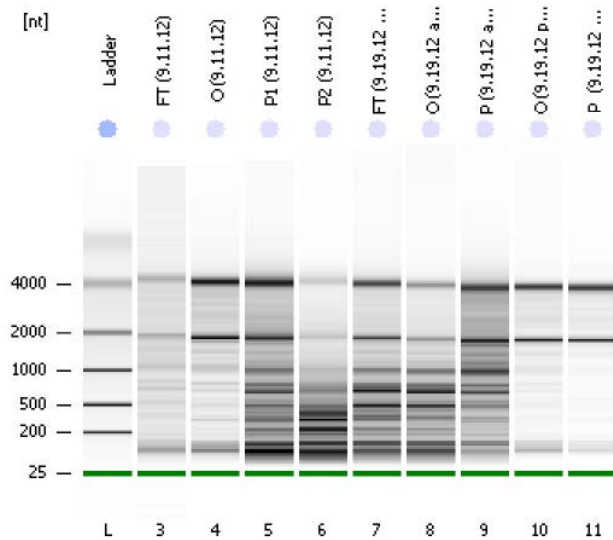


Figure 2. Electrophoresis showing quality of normal tissue brushings. The results are variable, suggesting that ~50% of collected samples will be suitable for analysis.

Task 3c. Process all specimens on various genomic platforms

We now have a collection of 100 qualified samples. We have extracted nucleic acids for all qualified specimens. We will run them all in a single batch. We had planned on using various array platforms for genomic analyses. Since the proposal has been submitted most genomic studies of this scale have moved toward more modern methods of measurement. For this reason, we will measure gene expression with RNA sequencing on an Illumina HiSeq machine instead of using the Affymetrix U133A 2.0 transcription array. The RNAseq provide better coverage and direct, rather than indirect, assessment of gene expression. We will use the Affymetrix 6.0 SNP array instead of the oligonucleotide Agilent 1M CGH array for somatic copy number alterations, due to familiarity and reliable performance of the Affymetrix array. We will measure microRNA expression with the Nanostring platform as it also provides better coverage and direct, rather than indirect, assessment of gene expression when compared with the Agilent Human miRNA Microarray. Sufficient nucleic acids will be stored for technical validation procedures.

Task 3d. Analyze data on each platform according to proposal

Pending

Task 3e. Write manuscript and publish results

Pending.

Section III – Problem Areas for Project 2

A1. We initially encountered some difficulty in determining how to characterize specimens for Task 1 when tumor was present within the fallopian tube. We have decided that these tumors should be classified as tubal carcinomas when the bulk of disease is clearly within the tube. We noted that in many cases there was a predominance of tumor at the fimbria and standard diagnostic criteria would classify these cases as tubal carcinoma, which is likely the result of overgrown STIC lesions. In the event that cases cannot be correctly classified, we exclude them from the study population.

A3. We have been slow to accrue the normal tissues from all centers including MSKCC. However, we had been focusing our efforts on completing the collection and processing of the tumor samples so that we can begin to run them as a single batch. This is now complete and we will re-focus our efforts on collecting the normal control samples. Being solely a comprehensive cancer center, we see few non-cancer cases from which we can obtain normal tissue brushings. We will need to rely on our

collaborators at JHU and Toronto to assist with this collection. Since training our colleague at JHU we have received further specimens. One of our recently graduated fellows has also joined the staff at JHU and we now have close ties with two collaborators to help with collections.

B1. We have not yet requested cases from outside centers for Task 1. The reason we have not yet done this, is that we wanted to first collect the outside material for Task 3 which had been given priority. Since the processing of Task 3 is now complete we can return to devoting effort toward the completion of Task 1 as far as case collection from outside consortium centers (JHU, Toronto, and Yale [Task 1]).

Section IV – Work for next reporting period for Project 2.

Task 1 – We will collect additional cases for other consortium sites.

Task 2 – We will use the available MSK data to evaluate whether the presence of a STIC is associated with different clinical manifestations and /or outcome compare to those patients in whom a STIC was not identified.

Task 3 – We will begin the genomic analysis for the 100 qualified cases.

We will continue to accrue additional normal brushings from MSK and Hopkins with greater effort.

Other activities:

Project 1:

We have provided 15 cases from MSK for the work under Project 1. We have provided clinical data and biospecimens.

Project 5:

- We have submitted clinical and epidemiologic data for 45 cases, of which 6 were redacted due to updated inclusion criteria.
- We have collected data on 95 additional cases and will submit those this month to the coordinating center.
- We have identified 33 consecutive cases for Project 5 as requested by the Project 5 PI for pilot studies.
- We are awaiting a final version of a prospective survey to include in our prospective biospecimen acquisition protocol so that we can collect prospective epidemiologic data for Project 5.

PROJECT 3. Identification of molecular changes preceding STICs in FTE from high-risk women using in vitro and in vivo models

Investigators: Shaw, Gauthier, Shih, Berman (consultant: Cho; collaborator: Morin)

Research site: University of Toronto

In this project we will determine if the expression profiles of anatomically high risk FTE (fimbrial) from women at high genetic risk (BRCA1 mutation carriers) differs from the FTE profiles from women at low risk of HGSC, and we propose that these changes may play key roles in the earliest events of serous carcinogenesis. To this end, we will use a molecularly defined system to sequentially express ovarian cancer-associated genes including those identified in this project into ovarian surface epithelium (OSE) as well as fimbrial FTE to determine a) if FTE is more prone to neoplastic transformation and b) if the FTE-derived tumors more closely simulate HGSC than OSE-derived tumors.

Specific Aim 1. *Detect and select genes differentially expressed in morphologically normal fimbrial FTE from women at high genetic risk of HGSC.*

Specific Aim 2. *Model alterations associated with normal FTE from high-risk women and STIC in vitro and in vivo.*

Section II. Progress to Date

Task 1.

1. Establish expression profiles of fallopian tube epithelium from BRCA1 mutation carriers and controls, and of serous cancers in mutation carriers.

1a. Histologic review of ovaries and fallopian tubes from prophylactic salpingo-oophorectomy specimens, matching controls, and cancers (months 1-6).

Progress: To date, we have collected and processed over 200 formalin-fixed and/or cryopreserved cases of fallopian tube and fimbriae specimens. These cases include samples from BRCA1 and BRCA2 mutation carriers undergoing prophylactic surgery, patients undergoing debulking surgery for High Grade Serous Carcinoma, and patients undergoing salpingo-oophorectomy for non-malignant reasons. An integral aspect of Project 3 *Specific Aim 1* is to determine the relationship between hormonal response and BRCA mutation status in the normal fallopian tube epithelium. As a result, a significant effort has been placed on determining the menstrual status of samples collected – this has included reviewing the endometrium of corresponding samples when available.

1b. Selection of a minimum of 32 fallopian tubes from *BRCA1* carriers, 32 from normal controls, 16 STICs, and 16 serous cancers from *BRCA1* mutation carriers (months 1-6).

Progress: We have completed histological reviews of fallopian tubes from *BRCA1* mutation carriers, along with matching controls and cancers and identified 84 cases that can be used for gene expression profiling. One of the key questions within this aim is whether the gene expression differences identified at the ampulla between *BRCA1* and non-*BRCA1* mutation carriers is similar to differences at the distal end of the fallopian tube – the fimbria. We have determined therefore, to answer the ampulla versus fimbria conundrum, it would be technically robust to use cryopreserved ampulla and fimbria from non-*BRCA* mutation carriers with known ovulation cycle status, using techniques our laboratory has used successfully in previous studies. We have completed the selection of 6 luteal phase and 6 follicular phase cases containing both distal end fallopian tube and matching fimbriae tissues for a total of 24 samples (Table 1). For the paraffin samples we have completed the selection of all *BRCA* and normal control fimbriae tissues and all HGSC samples (Table 2).

1c. Microdissection of selected tissue samples (months 3-10).

Progress:

A). Micro-dissection of selected cryopreserved tissue samples

For laser capture micro-dissection, 10-µm frozen sections were cut onto PEN membrane slides (Leica), and immediately stored on dry ice. An entire cross-section of fallopian tubes for each case underwent LCM, and most of the epithelium for each cross-section was captured, with approximately, 3-6 sections were cut per sample. Two anatomical regions (fimbria and ampulla proximal to the fimbria) were assayed for each case. Additionally, 5-µm sections were cut at the beginning and end of each block to ensure proper tissue orientation and histology. The Leica LMD 6500 with CTR MIC controller was used to obtain epithelial cells. Total RNA was isolated using the RNeasy Micro Kit (Qiagen Inc). Quality and quantity of RNA was confirmed using the Agilent 2100 Bioanalyzer RNA 6000 Pico LabChip kit (Agilent Technologies) and NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies), respectively, before inclusion in the study. Ten nanograms of total RNA from each study case will be reverse transcribed and linearly amplified using the Ovation Amplification System V2 kit (NuGEN Technologies). Quality and quantity of the purified labeled cDNA product were confirmed before hybridization to Affymetrix GeneChip Human Gene 2.0ST arrays (Affymetrix, Inc.) according to manufacturer's instructions. To date we have completed micro-dissection of 19 of the 24 cryopreserved samples and will finish the outstanding 5 cases in the next few weeks.

B). Microdissection of selected paraffin tissue samples

We have optimized laser capture microdissection (LCM) on paraffin embedded tissues and determined the range and quality of RNA concentration and quality. We were able to isolate 10ng/ul from 6 sections of a luteal phase fimbria. For laser capture microdissection, 10-µm sections were cut onto PEN membrane slides (Leica), and immediately stored -80C. An entire cross-section of fimbria for each case underwent LCM, and most of the epithelia for each cross-section were captured, with approximately, 7-10 sections were cut per sample. The

Leica LMD 6500 with CTR MIC controller was used to obtain epithelial cells. Total RNA was isolated using High Pure FFPE RNA Micro Kit (Roche). Quality and quantity of RNA was confirmed using the WG-DASL HT Assay (Illumina) and Quant-iT RiboGreen RNA Assay Kit (Invitrogen), respectively, before inclusion in the study. One hundred of total RNA from each study case will be reverse transcribed and linearly amplified using the Ovation Amplification System V2 kit (NuGEN Technologies). Quality and quantity of the purified labeled cDNA product will be confirmed before hybridization to Illumina DASL arrays (Illumina) according to the manufacturer's instructions. Additionally for the paraffin tissues, fimbriae was micro-dissected from 5 luteal and 5 follicular samples of the projected total and these samples are ready for submission for the Illumina DASL arrays (Table 3).

1d. Profile gene expression of cellular samples (months 10-12).

Progress: The cryopreserved micro-dissected samples will be submitted to the TCAG microarray facility within the next couple of weeks. For the paraffin samples we project the submission of all samples to the microarray facility within the next 6 months.

1e. Analysis of expression profiles with focus on genes differentially expressed in *BRCA1* mutation carriers (months 12-20).

Progress: We project completion of analysis for the cryopreserved microarray data within the next 6 months.

Task 2. Validate mRNA and protein expression of selected classifier genes.

2a. Creation of Tissue Microarrays, including benign and malignant tubal/ovarian tissues from mutation carriers and non-carriers (months 12-18)

Progress: In preparation for targets from the genomic profiling of STICs and fimbria gene expression arrays, we have created one additional fallopian tube tissue microarray (in addition to the ones from previous reports) containing BRCA1, BRCA2 and non-BRCA cases, utilizing the following exclusion criteria:

- Systemic steroid treatment for other conditions (ie SLE/ankylosing spondylitis), history
- Recent breast cancer patients (<2 years) with unknown adjuvant treatment history
- Hormone therapy treatment
- Patients on HRT at time of surgery
- Unknown menstrual cycle status at time of surgery

The tissue microarray includes the following permutations (2 cores per case, 1.0mm diameter per core):

- Luteal: BRCA1 (N=10) and BRCA2 (N=10)
- Follicular: BRCA1 (N=10) and BRCA2 (N=10)
- Non-BRCA: Luteal (N=10) and Follicular (N=10)
- Post-menopause: BRCA1 (N=5), BRCA2 (N=5), Non-BRCA (N=5)

2b. Validation of selected genes by quantitative RT-PCR (months 20-30).

Progress: Pending

2c. Selection of antibodies for validation of protein expression (months 24-30).

Progress: Pending

2d. Data analysis (months 30-36).

Progress: Pending

2e. Validation of selected proteins by immunohistochemistry in tubal cancer precursors and occult serous carcinomas (months 30-36).

Progress: Pending

Task 3. Model alterations associated with high-risk tubal epithelium and tubal intraepithelial carcinomas, in vitro.

3a. Isolate and propagate primary FTE (months 1-6).

Progress: In previous reports we have showed the ability to isolate and propagate FTE with more than 10 primary FTE cell lines established from different patient samples.

3b. Characterize primary FTE growth kinetics and marker expression (months 1-8).

Progress: We have isolated and characterized primary FTE to establish an in vitro model system for the study of early events in HGSC. FTE propagate in culture for ~5 population doublings before reaching an irreversible replicative senescence. We find that inactivation of retinoblastoma (Rb) or p53 alone or a combination of both,

with and without hTERT, or SV40 large T, respectively, extends FTE past 15-30 population doublings without replicative senescence.

3c. Generate pre-malignant (immortalized/non-tumorigenic) FTE.

Progress: More than 35 FTE cell lines including 5 from mutation carriers have been established containing a number of different permutations of molecular alterations. We have generated FTE pre-malignant cells using two approaches using HPV16-E7 and DNTP53 with and without hTERT expression. This approach has been successful in generating immortalized human epithelial cells. These cell lines have been carefully characterized in terms molecular alterations during construction, morphological appearance in 2D monolayer, shared epithelial/stromal markers with corresponding fallopian tube tissue, growth ability by population doublings and anchorage independent growth (Figure 1).

3d. Define phenotypes associated with pre-malignant FTE-BRCA vs FTE-nonBRCA (months 12-30).

Progress: We are in the process of characterizing FTE-BRCA and non-BRCA cell lines, by performing alterations in genes known to be differentially expressed between BRCA1 heterozygotes and normal controls, followed by in vitro assays used to identify specific phenotypes.

LKB1 and p53

LKB1, a candidate gene associated with loss of cellular polarity, is significantly decreased in FTE-BRCA and HGSC and associated with frequent deletions in the tumors (19/75 of samples run on WG 6.0 SNP array). Frequent decrease in LKB1 differentially found in HGSC, with other epithelial ovarian cancer histotypes showing high levels of protein expression (Figure 2). IHC in 15 STICs shows 100% loss of LKB1 protein expression compared to normal FTE, further indicating that alteration of the gene occurs early (Figure 3). LKB1 loss in primary FTE cells immortalized by p53 inhibition, leads to cellular senescence (Figure 4). However, in combination with cyclin E over-expression there is change in phenotype (Figure 5). Cyclin E over-expression and LKB1 loss synergize allowing for bypass of senescence leading to an increase in proliferation and anchorage independent growth (Figure 5). These results suggest the importance of LKB1 inactivation is early in carcinogenesis.

FTE cells were grown in an air-liquid interface culture, which mimics the lumen of the fallopian tube. Cells were infected with a p53DN transgene by a lentiviral vector to recapitulate the p53 signatures observed in the fimbria (Figure 6). Cells grown under these conditions were characterized by immunohistochemistry and immunofluorescence, for the presence of cilia (acetylated-tubulin; ac-tubulin), polarity (ezrin – apical to basal polarity), and E-cadherin. These cells maintain markers commonly identified in vivo (Figure 6). Additionally, as seen in vivo, in p53 signatures, these cells showed a decrease in ciliated cells, determined by scanning electron microscopy (Figure 7). Quantitative analysis of ac-tubulin, a marker of ciliated cells, by four independent observers, identifies a statistically significant decrease in ciliated cells when mutant p53 is overexpressed ($p < 0.01$) (Figure 7). These observations indicate that these cellular permutations might lend mechanistic understanding to the development of the 'p53 signature' via two routes – expansion of secretory cells with a p53 mutation or expansion of ciliated cells that have lost their cilia and up-regulated Pax8 (this hypothesis still needs to be tested).

3e. Determine if candidate alterations (defined in Project 1, 2) modulate pre-malignant phenotypes, using cell culture and CAM assays (months 12-36).

Progress: In addition to LKB1 alterations, specified in section 3d we have also analyzed alterations in CEBPD. CEBPD represents a master regulator of gene transcription primarily through STAT3 activation in many tumor types. In a previous publication we found CEBPD to be significantly increased in FTE-BRCA1 and specifically in the luteal phase of the menstrual by gene and protein expression⁴. CEBPD is commonly induced by stress and thus explains its higher expression in the luteal phase, which is associated with increased DNA damage. Additionally, studies in breast cancer showed that CEBPD can interact with FANCD2, which is involved in DNA repair along with BRCA1 and BRCA2⁵. Interestingly, CEBPD is down-regulated in over 60% of HGSC analyzed, an observation which also histotype specific as LGSC tumors show high expression levels (Figure 11b). To determine how early CEBPD is deregulated we performed IHC on 15 STIC cases. Results show moderate protein expression in most STICs with expression maintained exclusively in non-proliferating cells, measured by Ki67, whilst expression loss is observed in the corresponding HGSC (Figure 11a). This suggests that CEBPD regulates differentiation and cell-cycle regulation in the FTE possibly through the G2/M check point (as it is also co-upregulated with GADD45 from our previous work. We have begun to study the effect of loss and gain-of-function of CEBPD in FTE cell cultures in order to understand the link between BRCA and CEBPD and the effect that increased CEBPD levels may have in BRCA mutation carriers.

3f. Determine if candidate alterations (defined in Project 1,2) modulate in vivo malignant phenotypes (months 36-48).

Progress: The combinations of gene alterations in FTE cell lines harboring p53, RB1, hTert, and hRAS or Myc1 or PIK3Ca, were not sufficient to develop tumors in mice during a 6 month process.

To assess in more detail, the ability of molecular alterations to promote metastatic colonization we have also assessed the ability of high priority cell lines to colonize the omentum (milky spots), the primary site of metastasis and the first step of development of peritoneal disease. Milky spots represent organized structures of omental fat found in humans, rodents, and other mammals and are comprised of immune, stromal, and progenitor cells localized to dense vascular networks. To test the colonization ability for our established fallopian tube cells, 5×10^6 cells of the FTE-p53DN-E7-hTert-hRas cell line were injected into 6-8 week old female athymic nude mice. 7 days post injection, omenta were collected and processed for immunohistochemistry. Serial sections were stained for H&E, Cytokeratin 18 and PAX8 (Figure 8). We are able to show that although these cells are not able to develop a tumor in the mouse, these cells do possess certain metastatic characteristics as they can colonize milky spots and importantly they maintain epithelial markers present in the fallopian tube (Figure 8).

Thus far 3 independent groups have published xenograft mouse models using fallopian tube cells with various genetic permutations^{1,2}. For an initial proof of principle we have established cell lines containing PP2A inactivation, in addition to the initial molecular alterations (p53, RB1, hTert, Ras and Myc). These cell lines are currently being tested for ability to colonize the peritoneal cavity in the mouse.

Additional candidates for drivers of malignant phenotypes relevant to HGSC could be identified by: a). determining large spanning frequent gene amplifications/deletions, gene expression regulation, and epigenetic regulation as has been shown by the recently published work on serous carcinoma by the TCGA³; b). assessing additional alterations present in the STICs; c). determining markers with clinical outcome significance. Immunohistochemical analyses in 15 STICs of different biomarkers were analyzed. P16 and RB1 IHC have identified non-redundant retinoblastoma pathway alterations that occur at different frequencies across STICs: 60% P16 homogeneous expression, 30% RB1 deletion (Figure 9). Also, these non-synonymous alterations stratify HGSC samples with different clinical outcome parameters (Figure 10). These differences suggest the possible use of different candidate drivers of malignant transformation to reflect subtypes of HGSC.

3g. Determine differential malignant phenotypes in FTE vs OSE derived tumors in vivo (months 48-60).

Progress: Pending

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- In vitro loss of p53 in primary fallopian tube cell lines mimics in vivo data showing statistically significant loss of ciliated cells
- Determination of malignant characteristics of primary FTE cells with limited gene alterations (p53DN-E7-hTert-hRas cells). These cells have the ability to colonize milky spots but are not tumorigenic in the mouse peritoneal cavity
- LKB1 loss is identified as an early alteration, is frequently present in STICs, and is functionally involved in morphological changes in primary FTE cell lines. Cyclin E and LKB1 synergize leading to cellular transformation and preliminary malignant characteristics
- Characterization of retinoblastoma pathway alterations identify Non-redundant deregulations that are present in STICs and may reflect different HGSC molecular subtypes to be tested using mouse models

REPORTABLE OUTCOMES:

Publications

1. Retinoblastoma Pathway Deregulatory Mechanisms Determine Clinical Outcome in High Grade Serous Ovarian Carcinoma. Anca Milea, Sophia George, Donco Matevski, Haiyan Jiang, Mary Madunic, Hal Berman, Mona Gauthier, Brenda Gallie, Shaw Patricia. *Modern Pathology*. In press 2013.
2. Sophia HL George and Patricia Shaw. BRCAness and the fallopian tube epithelium. *Frontiers in Oncology*. In preparation.
3. Sophia HL George, Anca Milea, Ramlogan Sowamber, Rania Chehade, Alicia Tone, Mona Gauthier and Patricia Shaw. Loss of LKB1 Protein Expression is Frequent in Serous Carcinoma. *In preparation*

Abstracts

1. Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. The role of estrogen receptor signaling in serous ovarian cancer. *Cancer Research* August 14, 2013 73:4765; doi:10.1158/1538-7445.AM2013-4765
2. Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. STK11 and p53 synergy in fallopian tube epithelial and high-grade serous cancer (September 2013). *Advances in Ovarian Cancer Research: From Concept to Clinic* conference.

Oral Presentations:

Sophia HL George Origins of ovarian cancer, the biology of premalignancy, and cancer health disparities. AACR The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved on December 6 - 9, 2013 in Atlanta, GA.

Patricia A Shaw LKB1 – Does it have a role in early ovarian cancer? 4th Annual Ovarian Cancer Symposium Prevention and Early Detection of Ovarian Cancer. May 2013, Memorial Sloan Kettering Cancer Center, New York, NY

Patricia A Shaw Pathology of Ovarian Cancer. Breast and Ovarian Cancer in the Caribbean: Prevention, Diagnosis and Treatment. Women's Cancer in the Caribbean Workshop, Dominica. May 2013, Roseau, Dominica

George HL Early events in High Grade Serous Cancer Development. HL Women's Cancer in the Caribbean Workshop, Dominica. May 2013, Roseau, Dominica

Section III. Problem Areas for Project 3

We do not identify potential problems at this moment. These scientific results have important implications for the development of HGSC. The in vitro results using p53 and LKB1 inhibition identify a functional synergy between these two alterations leading to significant decrease in ciliated cells. These cells maintain fallopian tube epithelial markers such as pax8 and E-cadherin, identified in normal fallopian tube epithelium. These results indicate that our in vitro model closely mimics the normal/p53 signature in vivo conditions and that LKB1 inactivation represents a very early event in tumor formation.

Section IV. Future Work in Project 3

Further immunohistochemical characterization of STICs will be performed because it is important in identifying candidate genes that may be utilized for metastatic cell transformation. For example, analysis of the Rb pathway in STICs has revealed non-synonymous alterations that go on to stratify tumors with clinical outcome differences. Furthermore, we will perform in depth analysis of primary fallopian tube cell lines with different molecular alterations, has revealed that although certain metastatic characteristics may be adopted, such as increased proliferation, anchorage independent growth and colonization of milky spots, these cells are not necessarily able to form tumors and colonize the mouse peritoneal cavity.

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1. Jazaeri AA, Bryant JL, Park H, *et al.* Molecular requirements for transformation of fallopian tube epithelial cells into serous carcinoma. *Neoplasia* 2011; 13(10):899-911
2. Karst AM, Levanon K, Drapkin R. Modeling high-grade serous ovarian carcinogenesis from the fallopian tube. *Proc Natl Acad Sci U S A* 2011; 108(18):7547-52.
3. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011; 474(7353):609-15.
4. George SH, Greenaway J, Milea A, *et al.* Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. *J Pathol* 2011; 225(1):106-17.
5. Wang J, Sarkar TR, Zhou M, *et al.* CCAAT/enhancer binding protein delta (C/EBPdelta, CEBPD)-mediated nuclear import of FANCD2 by IPO4 augments cellular response to DNA damage. *Proc Natl Acad Sci U S A* 2010; 107(37):16131-6.

APPENDICES:

1. Retinoblastoma Pathway Deregulatory Mechanisms Determine Clinical Outcome in High Grade Serous

Ovarian Carcinoma. Anca Milea, Sophia George, Donco Matevski ,Haiyan Jiang, Mary Madunic , Hal Berman, Mona Gauthier, Brenda Gallie, Shaw Patricia. *Modern Pathology*. 2013.

2. Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. The role of estrogen receptor signaling in serous ovarian cancer. *Cancer Research* August 14, 2013 73:4765; doi:10.1158/1538-7445.AM2013-4765

SUPPORTING DATA:

Table 1: Samples to be used on AffymetrixGeneChip Human Gene 2.0ST N=24

	Fimbria Luteal	Fimbria Follicular	Ampulla Luteal	Ampulla Follicular	Total
N	6	6	6	6	24

Table 2: A summary of FFPE cases selected for LCM and DASL gene expression array.

	BRCA1 Luteal	BRCA1 Follicular	Normal Luteal	Normal Follicular	Total
N	12	15	14	20	61
HGSC					16

Table 3. A summary of FFPE samples completed to date.

Samples	Concentration (ng/microL)	Volume (microL)	RIN
Normal_Luteal_1	7.1	12	1.5
Normal_Luteal_2	15.6	12	2.1
Normal_Luteal_3	20.1	12	2.5
Normal_Luteal_4	14.1	12	2.4
Normal_Luteal_5	18.1	12	2.1
Normal_Follicular_6	14.2	12	2.5
Normal_Follicular_7	20.1	12	2.2
Normal_Follicular_8	13.6	12	2.5
Normal_Follicular_9	12	12	2.2
Normal_Follicular_10	15	12	2.3

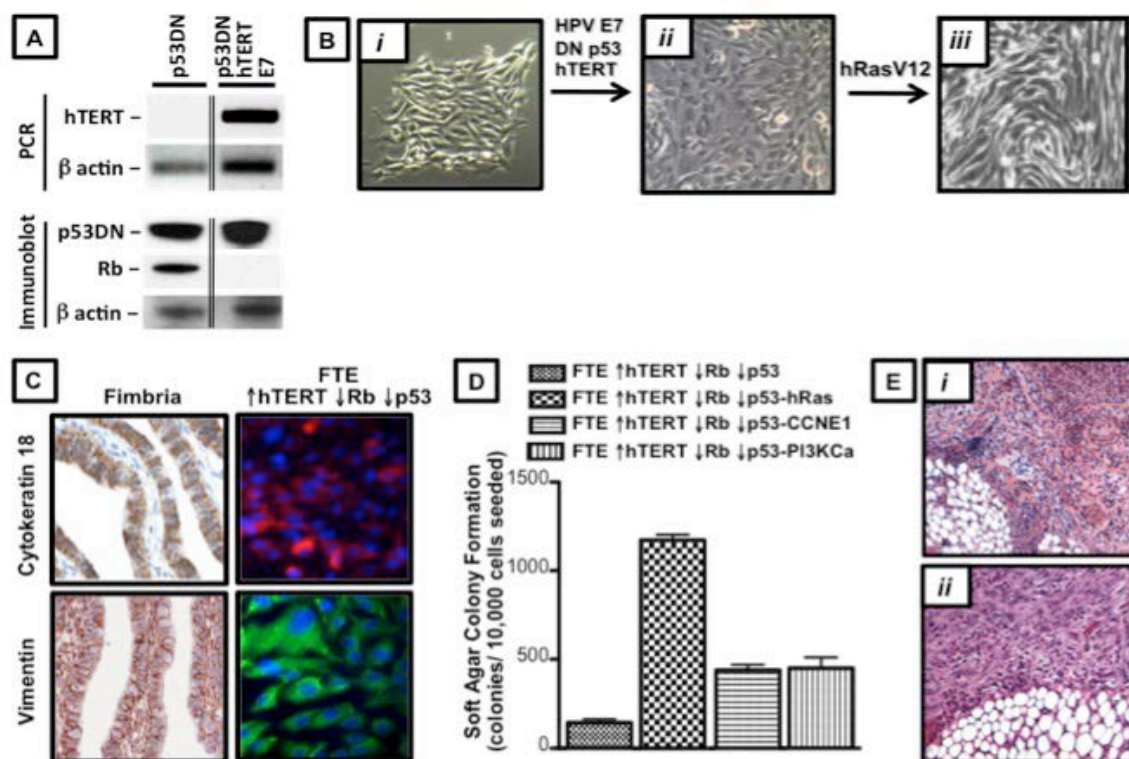


Figure 1. FTE-derived cell lines which model HGSC colonize the omentum. **A** Example of molecular assessment of cell lines during construction; **B**. Appearance of cells growing in monolayer culture: *i* primary FTE; *ii* FTE ↑hTERT ↓Rb ↓p53; *iii* FTE ↑hTERT ↓Rb ↓p53 cells expressing either hRasV12, CCNE1, or PIK3CaH1047R all have the same appearance in tissue culture (representative data from hRasV12 cells is shown); **C** FTE ↑hTERT ↓Rb ↓p53 cells continue to express cytokeratin 18 (red) and vimentin (green) just as normal FTE cells in the fimbria do; **D** Assessment of growth of cell lines under anchorage-independent conditions; **E** H&E stained tissue from omentum (*i*) and splenoportal fat (*ii*) at 7 dpi after injection of FTE ↑hTERT ↓Rb ↓p53-hRas cells in our standard metastatic colonization assay.

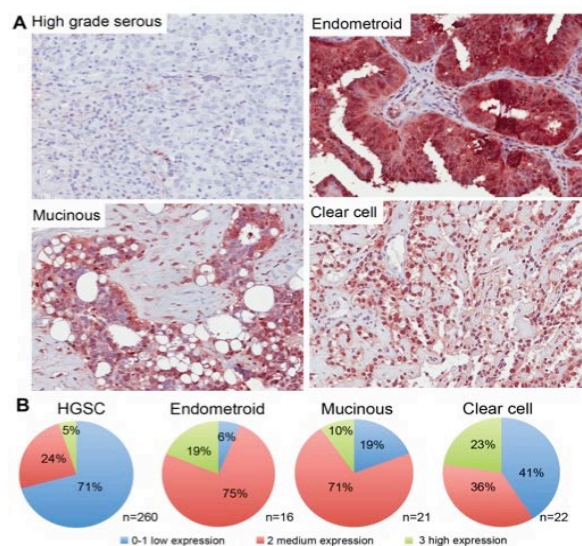


Figure 2. LKB1 immunohistochemical visualization and quantification identifies predominantly low protein expression (71%) in HGSC compared to other epithelial ovarian cancer histotypes.

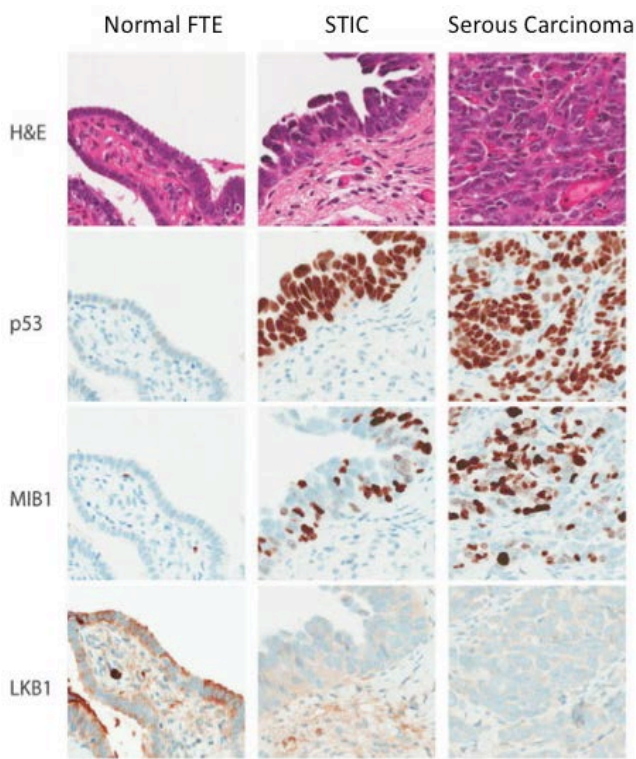


Figure 3. Visualization of LKB1 protein expression across normal FTE, corresponding STIC and HGSC indicating loss of LKB1 in the STIC.

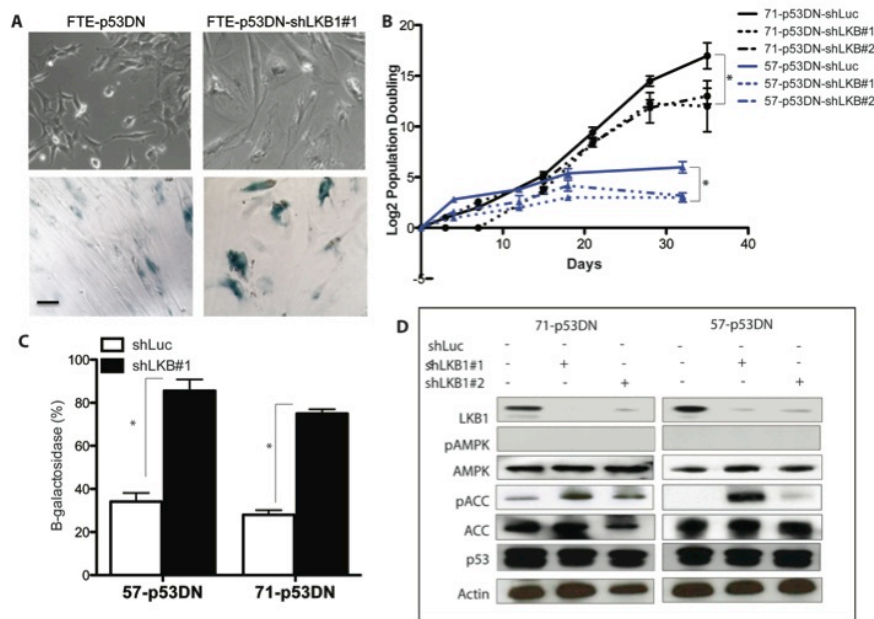


Figure 4. Characterization of FTE-p53DN and synergy between p53DN and LKB1 loss in 2D monolayer tissue culture conditions. Loss of LKB1 leads to cellular senescence, identified by A. flat vacuolated cells and increased b-galactosidase staining. B. Decreased proliferation and C. Statistically significant increase in β -galactosidase. D. LKB1 loss leads to AMPK pathway activation through increased phosphorylation of pACC.

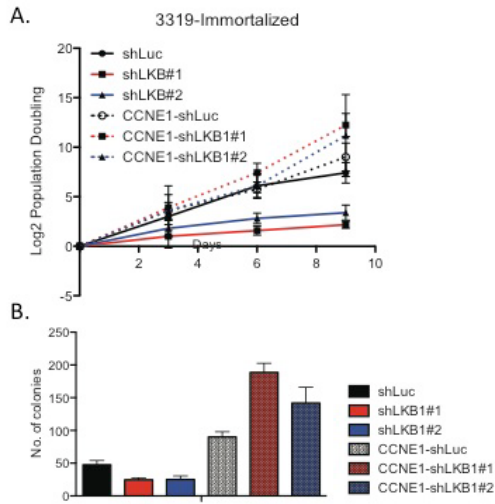


Figure 5. In vitro synergy between LKB1 loss and cyclin E over-expression. Cyclin E overexpression in cells with LKB1 loss leads to A. senescence bypass and increased proliferation and B. colony formation increase.

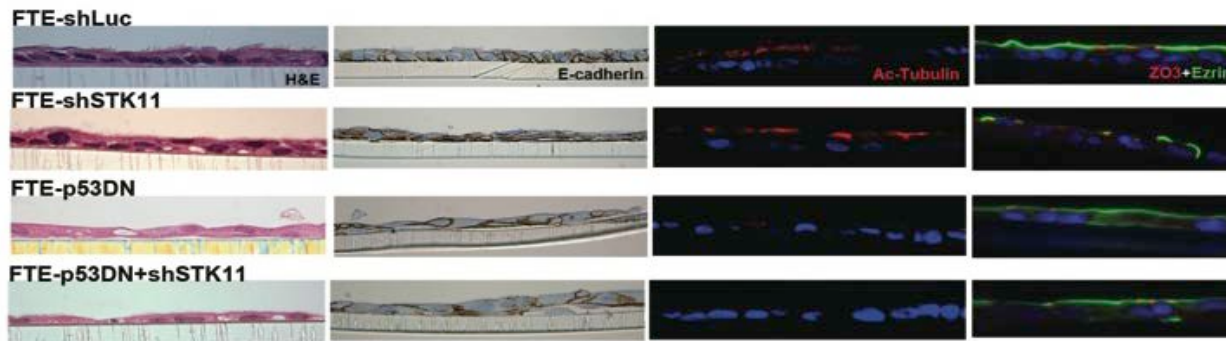


Figure 6. Characterization of control, FTE-shSTK11 FTE-p53DN and FTE-p53DN+shSTK11 cells grown in polarized tissue conditions indicate loss of ciliated cells in p53DN cells identified by low Ac-tubulin staining.

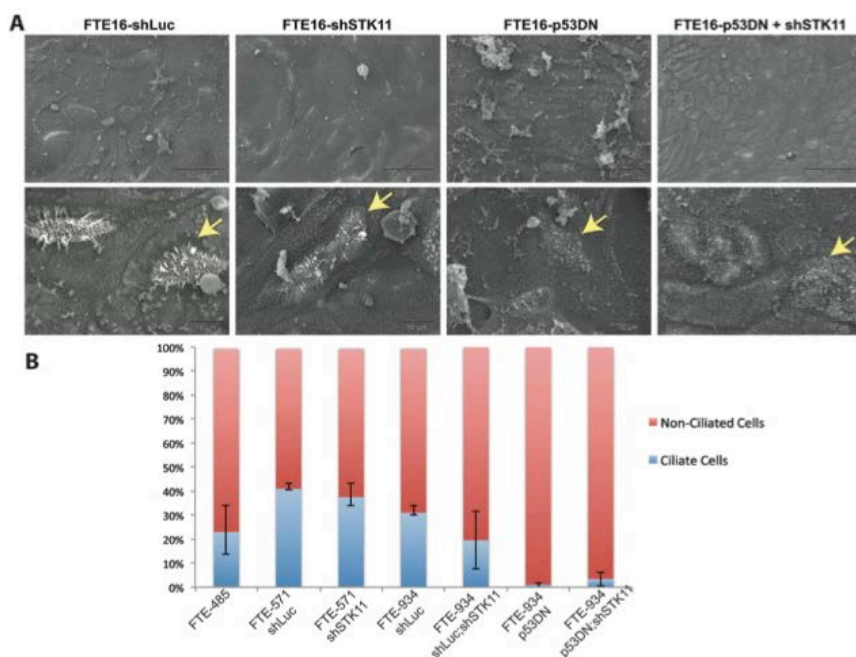


Figure 7. A. Visualization and B. quantification of decreased ciliated cells in primary FTE cells immortalized by p53 inhibition with LKB1 loss compared to p53 inhibition alone.

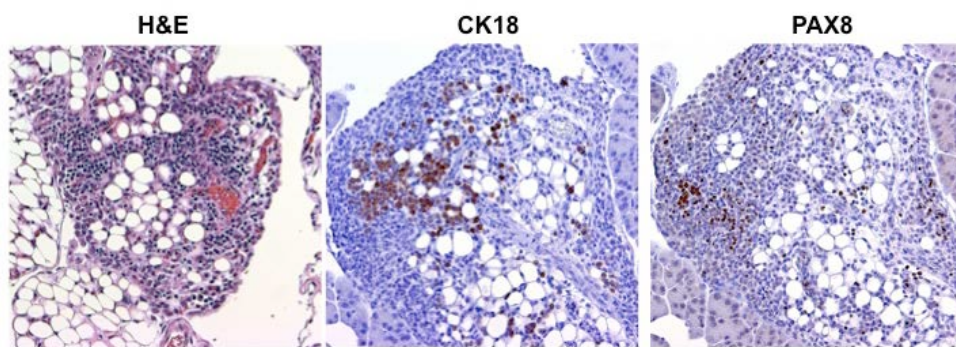


Figure 8. 5×10^6 imFTE (hRAS) were injected into 6-8 week old female athymic nude mice. 7 days post injection (dpi), omenta were collected and processed for immunohistochemistry. Serial sections were stained for H&E, Cytokeratin 18 (DAKO) and PAX8 (ProteinTech).

Figure 9. p16/RB1 immunohistochemistry patterns shared across serous tubal intraepithelial carcinomas and high-grade serous carcinoma (20x magnification). A). p16 homo/RB1+ subgroup identified in 47%. B). p16 hetero/RB1+ subgroup identified in 13% C). p16 homo/RB1- subgroup identified in 27%. The prognostic signature identified in invasive cancers is also found in concomitant serous tubal intraepithelial carcinoma. The definition of serous tubal intraepithelial carcinoma includes morphological characteristics and immunohistochemical staining of Ki67 and p53.

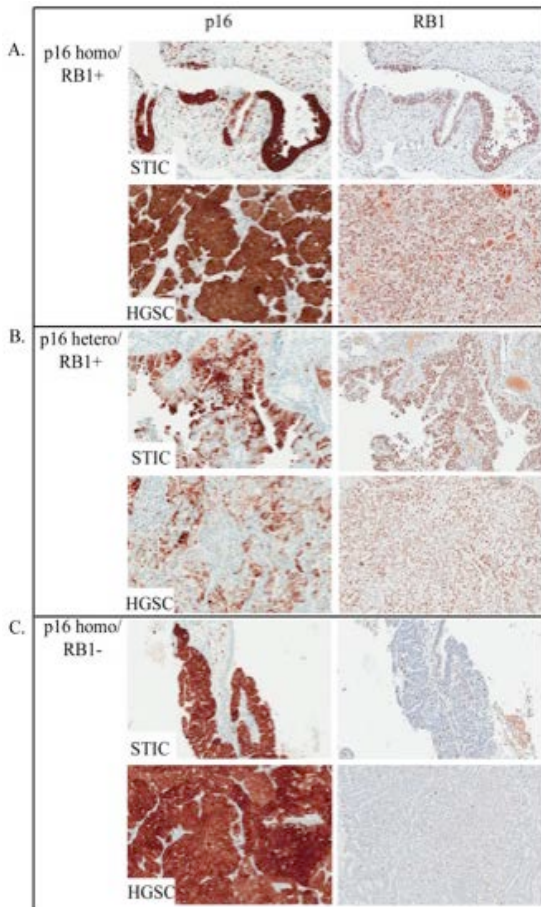
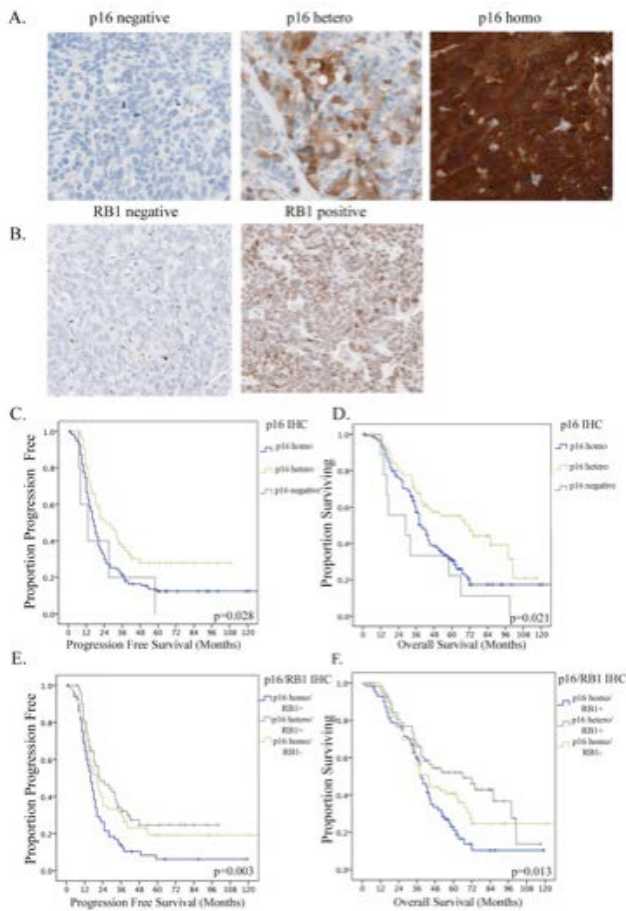


Figure 10. Kaplan-Meier curves of the differences identified in progression free survival and overall survival based on p16/RB1 immunohistochemical stratification. Representative images of immunohistochemical scoring for A). p16 – negative, heterogeneous, homogeneous; B). RB1 – negative and positive (20x magnification). Patient subgroups with differential p16 expression patterns showed significant differences in progression free survival and overall survival C) and D). Patient subgroups with differential p16/RB1 expression patterns also showed significant differences in progression free survival and overall survival E) and F).



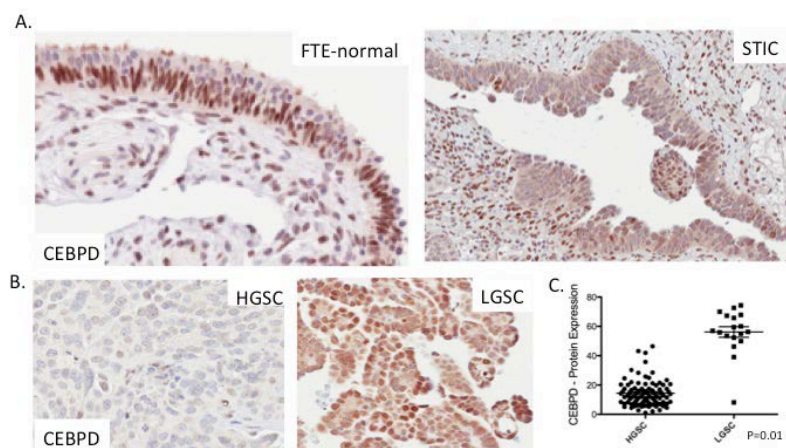


Figure 11. CEBPD alterations across histotypes and precursor lesions. Visualization (40x magnification) of CEBPD in A. FTE-normal and STIC, B. Expression is mostly lost in HGSC however is maintained in low-grade serous carcinoma, C. Quantification of CEBPD expression between HGSC and LGSC.

Project 4. Locate and characterize precursor lesions of “ovarian” cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in “ovarian” carcinogenesis using human tubal xenografts in nude mice.

Research site: Johns Hopkins University

Project Leader: Tian-Li Wang

Co-investigators: Ie-Ming Shih (JHU)

Section II. Progress to Date

Task 1. Locate the anatomic site and characterize the precursor lesions of “ovarian” cancer in the TP53^{-/-}/Rb^{-/-} mouse model.

Task 1a. Mouse breeding and genotyping (1-18 months)

Progress: This task has been finished. Please see the following tasks for details.

Task 1b. TP53/Rb KO mice study to locate the anatomic site of precursor lesions (1-15 months).

Progress: As discussed in the last progress report and in the DoD consortium investigator meeting at MSKCC (2013), we have finished the study related to TP53/Rb KO mice but failed to observe tumor phenotypes in at least 20 female mice for a period of time up to 16 months. Therefore, we have sought to use another mouse model. To this end, we have focused on Ovgp1 Tag mouse model and we have finished characterization of this model and found this is a highly appropriate model for the proposed studies in the Project 4. Please see the following attached manuscript draft for details.

Task 1c. Characterize the mouse precursor lesions using a variety of proposed methods (10-33 months).

Progress: As described in the last progress report, we have chosen to use Ovgp1 Tag mice as a transgenic mouse model for this study. We were able to demonstrate the precursor lesions including mouse STIC in this mouse model. Please see the following attached manuscript draft for details.

Task 2. Assess the biological effects of ovarian follicular fluid on human fallopian tube epithelium (FTE) and ovarian surface epithelium in a xenograft model.

Task 2a. Establish the human fallopian tube xenograft model (18-30 months).

Progress: We have started this task and found that human fallopian tube tissue fragments could survive in mouse peritoneal cavity for up to 10 days in 50% of xenografts. However, the mucosal surface appears to adhere severely to the abdominal organs. Currently, we are seeking a refined method to perform the xenografting to increase the survival of the human tissues in mice.

Task 2b. Collect human follicular fluid and primary characterization of the fluid (15-25 months).

Progress: Pending.

Task 2c. Assess the biological effects of ovarian follicular fluids on FTE and OSE in a xenograft model (30-48 months).

Progress: Pending.

Task 3. Determine whether oral contraceptives (OCPs) and NSAIDs reduce the morphologic and molecular changes that are associated with early “ovarian” carcinogenesis.

Task 3a. To assess whether OCPs decrease the frequency of precursor lesions and/or delay tumor development (24-48 months)

Progress: We have started this task by stopping ovulations in mice. We have included a total of 10 mice in this pilot study including 5 OCP treated and 5 control treated controls. We will report the findings in the next progress report.

Task 3b. To determine the effects of aspirin on reducing oxidative stress-induced molecular changes on human fallopian tube and/or on OSE (24-60 months).

Progress: As discussed with the advisory board members and other consortium investigators, we will change our priority by focus on determining if the statin drugs have a preventive effect on developing ovarian cancer and its precursor lesions using the OVGP1 mouse model. This is because statin drug has come to the research spotlight in cancer therapy and our preliminary results show a significant reduction of tumor progression in mouse tumor xenograft model. We are currently setting up experiments to test if statin drug can have an anti-tumor effect on the OVGP1 mouse model and we expect to report preliminary results in the next report.

Task 3c. Data analysis and preparation for publication (24-60 months).

Progress: We are going to submit the manuscript of the OVGP1 mouse model.

Section III. Problem Areas for Project 4

In task 2 as discussed above, we identified a potential problem in keeping the human fallopian tube xenograft alive for an extended period of time for our experiments. The tubal tissue fragments survived in mouse peritoneal cavity for up to 10 days in 50% of xenografts. However, the mucosal surface appears to adhere extensively to the abdominal organs and peritoneal wall. Currently, we are seeking a refined method to perform the xenografting to increase the survival and decrease the adhesion of the human tubal tissues in mice.

Section IV. Future Work in Project 4

By using Ovgp1 mouse characterized in Aim1, we plans to test agents including statin drugs that could be chemo-preventive for the ovarian carcinoma, especially inhibiting the formation of STIC, the precursor lesion of ovarian carcinoma as proposed. We will perform histological examination and immunohistochemistry using several tumor-associated markers including lamin C1, gH2AX, p53, and Ki-67. We will also determine the possible molecular mechanism how statin drugs inhibit tumor development. To this end, we will focus on the effects of statin on autophagy. The results from this preclinical study will pave the way to the future clinical trials of statin drug in ovarian cancer patients.

Attachment for Project 4 Report

A genetically engineered ovarian cancer mouse model based on fallopian tube transformation mimics human high-grade serous carcinoma development

Summary of the study:

Recent evidence suggests that human ovarian high-grade serous carcinoma (HGSC) originates from the epithelium of the fallopian tube (FT). However, most current mouse models are based on the transformation of the ovarian surface epithelium (OSE), as the prevailing view of ovarian cancer development, until recently, was that the OSE was the source of this neoplasm. Here, we report the extensive histological and molecular characterization of the mogp-TAg transgenic mouse, which expresses the SV40 large T-antigen (TAg) under the control of the mouse müllerian-specific *Ovgp-1* promoter. Histologic analysis of the fallopian tubes of mogp-TAg mice identified a variety of neoplastic lesions analogous to those described as precursors to human ovarian HGSC. We identified areas of normal-appearing p53-positive epithelium that are similar to “p53 signatures” identified in the human fallopian tube. More advanced proliferative lesions with nuclear atypia and epithelial stratification were also identified that were morphologically and immunohistochemically reminiscent of human serous tubal intraepithelial carcinoma (STIC), a potential precursor of ovarian HGSC. Beside these non-invasive precursor lesions, we also identified invasive HGSC in the ovary of 56% of the mice. Microarray analysis revealed several genes differentially expressed between the FT of mogp-TAg and wild-type C57/BL. One of these genes, *Top2a*, which encode topoisomerase II- α , was indeed shown by IHC to be concurrently expressed with elevated p53 and specifically elevated in mouse STICs, but not in surrounding tissues. Topoisomerase II α was also found elevated in human STICs, low-grade, and high-grade serous carcinoma. The mouse model reported here displays a progression from normal tubal epithelium to invasive HGSC in the ovary, and therefore closely simulates an emerging model for of ovarian HGSC pathogenesis in humans. This mouse therefore has the potential to be a very useful new model for elucidating the mechanisms of serous ovarian tumorigenesis, as well as for developing novel approaches for the prevention, diagnosis, and therapy of this disease.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy accounting for nearly 15,000 deaths annually in the US, yet the mechanisms of its origin, initiation, and progression remain unclear. In particular, the tissue of origin for epithelial ovarian cancer has recently become a matter of debate. Until recently, the ovarian surface epithelium (OSE), a relatively undifferentiated layer of mesothelial cells, has been thought to be the cell of origin for EOC, but this theory has several weaknesses (1, 2). First, it is puzzling that precursor lesions of ovarian high-grade serous carcinoma (HGSC), which accounts for the vast majority of EOC, have rarely been identified in the ovary. Second, the various types of EOC have a müllerian phenotype resembling the epithelia of the endometrium and fallopian tube (FT), which are derived from the müllerian ducts. In contrast, the ovaries do not develop from the müllerian ducts, but rather from the genital ridge. Third, in order to account for the similarity of HGSC, which resembles FT epithelium, as opposed to the mesothelial covering of the ovary, i.e., OSE, it has been argued that the OSE first undergoes a metaplastic change to tubal-type epithelium before undergoing malignant transformation. Recently, meticulous reexamination of the FT from women with a predisposition to ovarian cancer has identified malignant lesions in the FT, including early noninvasive precursor lesions of HGSC termed “serous tubal intraepithelial carcinomas” (STICs) (3, 4). Importantly, STICs have been linked to the development of serous ovarian cancer based on the observation that up to 60% of the patients with sporadic ovarian carcinomas also have STICs in their FTs (5). In addition, when HGSC and STICs coexist, they exhibit identical p53 mutations (5, 6). Moreover, morphologically normal-appearing areas of FT epithelium that exhibit p53 staining (termed “p53 signatures”) have been identified in the tubal epithelium and it has been proposed that they represent a precursor to STICs (7). Taken together, these recent findings suggest a model of ovarian tumorigenesis by which tubal epithelium gives rise to early intraepithelial carcinomas, which can then spread and implant on the ovary, where they grow and therefore appear to be primary ovarian neoplasms. This model recapitulates many of the pathologic and morphologic features of human HGSC, including the lack of precursor lesions in the ovary and the histological appearance of HGSC, which resembles FT epithelium.

Currently, the majority of genetically engineered mouse models of EOC target the transformation of OSE using various approaches (8, 9, 10: Laviolette, 2010 #2243, 11). In light of the evidence for the new paradigm described above, it is crucial to develop a mouse model of FT transformation. Here, we report the further characterization of a transgenic mouse, mogp-TAg, which spontaneously develops neoplastic lesions in the FT epithelium and the endometrium. We show that the mouse develops tubal lesions expressing many of the same markers as human STICs and that the transformed FT epithelium often invades the ovary to form tumors similar to human ovarian HGSC. Microarray analysis of the mouse tubal lesions identifies many altered genes and the expression of one of these genes, Top2A, is shown to be elevated in mouse and human STICs and may represent a novel early marker in ovarian cancer development.

Materials and Methods

Transgenic mouse

The generation of the mogp-TAg transgenic mouse has been described (12). Female mice for this study were euthanized at 4, 6, 7, 9, 12, and 13 weeks of age using inhalation of carbon dioxide followed by cervical dislocation. The animal study protocol was reviewed and approved by the National Institute on Aging Intramural Research Program animal care and use committee.

Tissues

Mouse ovary, FT, uterus, testis, prostate, liver, heart, intestine, and spleen tissues were collected and were either snap frozen, for RNA isolation, or fixed in 10% formalin for paraffin embedding. The formalin-fixed tissues were dehydrated through a graded series of ethanol and then embedded in paraffin and sectioned. Slides were stained with hematoxylin and eosin (H&E) for histological assessment. For the analysis of human STICs, archived formalin fixed embedded tissue cases were retrieved from the Johns Hopkins Medical Institutions. A total of 6 serous tubal intraepithelial carcinomas, 59 high-grade serous carcinomas, 8 low-grade serous carcinomas, and 8 normal FTs (controls) were included in this study. Tumor tissue was arranged in tissue microarrays, constructed using a manual tissue microarrayer. Triplicate 1 mm representative cores were obtained from each tumor block and arrayed on a recipient paraffin block. The collection of specimens was performed in accordance with an approved IRB. Commercial human Tissue microarrays (TMA) were purchased from US Biomax, Inc. that contained both normal FT (UTE601) and serous adenocarcinomas (BC111110).

RT-PCR

Total RNA was extracted from tissues using trizol according to the manufacturer's protocol (Invitrogen). Reverse Transcription of 1 µg of total RNA was performed using Taqman Reverse Transcription (Invitrogen). A normal mouse cDNA panel was also used (MNRT 101, OriGene). Primers were designed to amplify the mouse oviductal glycoprotein 1 gene (*Ovgp-1*): ctccacacttgccaaccg (*Ovgp1_m2-F*) and cataagacgtatggatgatgc (*Ovgp1_m2-R*). Levels of *Ovgp-1* cDNA were determined by quantitative real-time polymerase chain reaction using SYBR Green Mix (Roche) with mouse *Gapdh* normalization control as previously described (13). Data was analyzed using the comparative Ct method (14).

Immunohistochemistry

Mouse Immunohistochemistry was performed on 5 µm-thick sections after deparaffinization in xylene and rehydration through a decreasing alcohol series. Detection was accomplished by using the Labvision (Fisher) anti-rabbit protocol for the following antibodies: anti-Ki67 at 1:300 (Novus Biologicals); anti-p53 at 1:500 (Santa Cruz); anti-TAg at 1:500 (Santa Cruz); anti-H2AX at 1:500 (Cell Signaling); anti-PAX8 at 1:600 (ProteinTech); anti-Top2A at 1:200 (Epitome); anti-PBK at 1:25 (Cell Signaling) (15). Antibody signal was detected by oxidizing diaminobenzidine with hydrogen peroxide resulting in dark brown positive signal. Most sections were counterstained with hematoxylin and eosin (H&E).

For human immunohistochemistry, 4 µm-thick tissue sections were deparaffinized in xylene, rehydrated in graded alcohols, and heat induced epitope retrieval was performed by incubating in Trilogym (Cell Marque, Austin, TX, catalog # CMX833) in a steamer for 30 minutes. Endogenous peroxidase activity was blocked in a bath of 3% H₂O₂ solution in methanol for 15 min and washed in TBS-t. Non-specific protein-protein interactions were blocked with 200 µl/slide of block solution (10% Fetal Bovine Serum) for 30 min. The slides

were incubated overnight with primary monoclonal antibody against Topoisomerase II Alpha (Clone EP1102Y, dilution 1:200, Novus Biologicals, Littleton, CO, catalog # NB110-57623). EnVision™+/HRP, Rabbit polymer (Dako, Carpinteria, CA, catalog # K4003) was applied for 30 min and followed by 5 min of incubation with streptavidin peroxidase (Dako Liquid DAB+, Dako, Carpinteria, CA, catalog #K3468). The slides were counterstained in hematoxylin for 5 min (Sigma-Aldrich Corporation, St Louis, MO, catalog # HHS32), dehydrated in the graded ethanol (50%, 70%, 95%, 100%) for 5 min and incubated 3 times in xylene for 5 min each. IHC expression for Topoisomerase II Alpha was evaluated as the average percentage of nuclear positive tumor cells on 3 tissue cores.

Illumina microarray analysis

Total RNA was extracted from the FTs of three wildtype C57/BL mice as well as three mogp-TAg mice using Trizol (Invitrogen). RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA was prepared from 500 ng of RNA using the Illumina RNA Amplification Kit (Ambion) according to the manufacturer's directions. Hybridization to the MouseRef-8 Expression BeadChip (Illumina), washing and scanning were performed in the Illumina BeadStation 5006. Microarray data processing was done using Illumina Bead Studio software. Microarray analysis was performed essentially as described (16). Raw microarray data were subjected to filtering and z-normalization. Expression changes for individual genes were considered significant if they met 4 criteria: z-ratio above 1.5 (or below -1.5 for down-regulated genes); false detection rate <0.30; p-value of the pairwise t-test <0.05; and mean background-corrected signal intensity z-score in each comparison group is not negative (17). PCA analysis was conducted based on the above values using JMP software (SAS) to generate a 3D scatterplot with percent variance.

Statistical analysis

Comparisons of IHC outcomes between lesion groups were performed using the two-tailed unpaired t-test and p-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

TAg-expressing transgenic mouse

Gene expression profiling data shows that *OVGP1* is highly expressed in the epithelium of the human FT, but is found at much lower levels in other tissues (data not shown). In order to assess the value of the *Ovgp1* promoter in a mouse model of FT transformation, we examined the expression pattern of *Ovgp1* in mouse by RT-PCR. We examined 37 different mouse tissues and found *Ovgp1* expressed at very high levels in the FT (Supp Fig. 1). In fact, the levels of expression of *Ovgp1* were at least 100-fold higher in the FT compared to any other female tissue. The testis expressed the highest levels of *Ovgp1* in males, albeit at a much lower level than in FTs. We therefore hypothesized that the *Ovgp1* promoter may be used to specifically induce tumorigenesis in the FT through expression of the TAg. We therefore decided to further characterize the previously reported mogp-TAg mouse, which expresses TAg from the mouse *Ovgp1* promoter (12). Starting at approximately 6 weeks of age, tumors of the female genital tract were found in mogp-TAg females. As the females aged, we noticed increased inflammation of the uterus and an increase in size of both the uterus and the FTs. Females were euthanized at no later than 13 weeks of age to avoid of discomfort due to the enlarged uterus.

Histopathologic and immunohistochemical analysis of the FTs

In order to identify neoplastic lesions formed in the mogp-TAg model, mice of different ages were euthanized and the various tissues of the genital tract analyzed. Analysis of the FTs of mice 7-12 weeks of age, led to the identification of lesions that were overtly malignant, characterized by epithelial stratification and marked nuclear atypia, including mitotic bodies, enlarged nuclei, and irregular chromatin, resembling what has been described as STICs in human FTs (18: Carlson, 2008 #2141, 19)(Fig. 1). A human STIC is shown in inset for comparison. These lesions, which we refer to as mSTICs, were positive for p53 and expressed the TAg. Similar to what is observed in human STICs, the mSTICs were also highly proliferative as ascertained by Mib-1 (Ki-67) staining (Fig. 1).

Because of reports describing “p53 signatures” in human HGSC, (7, 19, 20) we searched for similar lesions in this mouse model. We identified areas in the FT mucosae of mice between 6-9 weeks of age that exhibited intense p53 staining, but did not show morphological signs of a carcinoma (Fig. 2). Again, TAg-staining was restricted to a small area that corresponded to the p53 staining. The mouse p53 signature shown in Fig. 2 exhibited high proliferation, as most of the cells present in the lesion were Mib-1 positive.

Invasive serous cancer in the ovary

In addition to tubal lesions, HGSC were detected in the ovaries of 56% of mogp-TAg mice examined between the ages of 8-10 weeks (Fig. 3). No carcinomas were found in younger mice. The invasive carcinomas were positive for TAg and p53, and exhibited a high proliferation index, as determined by Mib-1 staining. Interestingly, these carcinomas were positive for PAX8, a müllerian/FT marker, while the surrounding ovarian tissues, including the OSE, were negative. The invasive cancers also stained positively for γ -H2AX, a marker of DNA damage that has been shown to be positive in human STICs and in serous cancers (21). It is interesting to note that all mice with HGSCs also had p53 signatures and STICs in their FTs.

Gene expression profiling of neoplastic FTs

In order to better understand the mechanism of tumorigenesis in the FT of mogp-TAg mice at the molecular level, we compared gene expression profiles of FT from mogp-TAg mice with age-matched FT from WT mice. Microarray analysis identified a large number of genes whose expression was altered in mogp-TAg FT. Principal Component Analysis (PCA) was used to compare the two data sets, and this analysis revealed a distinct gene expression pattern between the FT from mogp-TAg mice and WT mice, with a 54.9% on the X axis of the scatterplot (Fig 4). There is little variance within the two groups (% variances of 9.3 and 14.2). We chose to focus our attention on genes that were elevated in the diseased FT, as we hypothesized that these genes may represent useful biomarkers for mSTICs and mouse p53 signatures, and possibly provide insight into the mechanisms of HGSC initiation. Table 1 lists the top 20 most highly up-regulated genes in mogp-TAg FT compared to WT FT. Six of these genes have previously been reported to be regulated by p53. Although this is not an unexpected finding, it highlights the prominent signaling mechanism in the transgenic FT tissue.

Topoisomerase II alpha is elevated in mouse high-grade serous lesions.

Tumorigenesis is initiated through p53 inactivation in this model, and p53 activation is a very early event in human serous carcinoma development as well. Therefore, we were particularly interested in the p53-regulated genes identified as altered in mogp-TAg FT, as they may represent genuine targets of p53 in serous carcinoma development. In particular, Pbk and Top2a, which have previously been implicated in human cancer (22, 23) were further investigated. We performed IHC for PDZ binding kinase (encoded by Pbk) and Topoisomerase II alpha (encoded by Top2a) in mouse sections from WT and mogp-TAg FT. PDZ binding kinase staining did not show reproducible patterns (data not shown), and was not investigated further. Topoisomerase II alpha exhibited strong staining in mSTICs and in the invasive portion of the tumor (Fig. 5). In particular, there was a striking correlation between the expression of Topoisomerase II alpha and the expression of p53 in these samples, suggesting strongly that Topoisomerase II alpha is regulated by p53 in this HGSC model.

Topoisomerase II alpha is elevated in human high-grade serous lesions.

In order to determine whether TOP2A was also expressed in human serous carcinoma, we stained human STICs and serous carcinoma for Topoisomerase II alpha expression (Fig 6). Human STICs and high grade serous carcinoma exhibited strong diffuse staining of Topoisomerase II alpha, while low grade serous cancer cells exhibited sparse staining at best and normal FT cells were negative. The transition in staining between normal FT and STIC was particularly striking, with a complete absence of staining in normal FT transitioning to strong staining in the STIC (Fig 6). Analysis of 8 normal human FT epithelium (NFT), 8 Low Grade Serous Carcinoma (LGSC), 59 High Grade Serous Carcinoma (HGSC), and 6 STICs demonstrated statistically significant differences in Topoisomerase II alpha expression between NFT and LGSC ($P=0.0001$), HGSC ($P=0.0039$), and STICs ($P=0.0013$) (Fig 6F). In addition, statistically significant differences were found between LGSC and both STICs ($P=0.0054$) and HGSC ($P=0.011$). There was no significant difference in the between STIC and HGSC ($P=0.40$) possibly suggesting lineage.

Discussion

Animal models of cancer represent an invaluable tool in the study of tumorigenesis and in the development of strategies for diagnosis and therapy. In particular, technological advances have allowed the development of genetically engineered mouse models that recapitulate genotypic and phenotypic features of human cancers (24). In ovarian cancer, multiple genetically engineered mouse models have been reported but, because of the prevailing view that HGSCs develop from the ovarian surface epithelium, most are based on the transformation of mouse OSE. Recently, a series of morphologic, immunohistochemical and molecular studies have suggested that epithelial cells in the distal tube (fimbria) give rise to ovarian (pelvic) HGSC (25, 26). According to this theory, secretory cells in the tube can clonally proliferate, and through a series of intermediate steps, give rise to intraepithelial serous carcinoma, which can either invade the tube before spreading to the ovary, or can shed cells that implant directly on the ovary and surrounding pelvic structures. Here, we present a transgenic model which expresses the TAg from the mouse *Ovgp1* promoter, a müllerian-specific promoter highly active in the sexually mature FT. The use of the TAg in the induction of FT carcinogenesis is appropriate, considering that p53 inactivation appears to be one of the earliest events in this cancer (26). This model is consistent with the FT origin hypothesis, as FT transformation using TAg leads to a series of lesions in the FT and to serous carcinoma in the ovary.

Examination of the FT of the mogp-TAg mice revealed the presence of several neoplastic lesions, including a mouse equivalent to the human STIC, mSTIC. In addition, we also found normal-appearing lesions that expressed p53, analogous to the p53 signatures in humans. The mouse p53 signatures were proliferative as determined by Ki-67 staining. Proliferative p53 signatures have been identified in human FTs and are thought to be an intermediate step between p53 signatures with low Ki-67 and STICs, which typically have a high proliferation index (26). We did not find non-proliferative p53 signatures in the samples we examined, but it will be interesting to examine additional tissues, possibly in younger mice, to determine whether such a benign lesion can be identified in this model. While the identification of these tubal lesions is interesting, the observation of HGSC within the ovary was particularly provocative. Nine of the 16 mice we examined contained a carcinoma implant in one or both ovaries. All were positive for PAX 8 expression, a müllerian marker found overexpressed in human ovarian HGSC (27), and for γ -H2AX, a marker of DNA damage also found expressed in serous cancer (28). Overall, these findings are consistent with the theory suggesting that human ovarian HGSC originates in the FT. While it is possible that the carcinoma observed within the mouse ovaries originated in the OSE, we believe it is unlikely for several reasons: 1) we did not observe TAg expression in any other areas of the ovary, including the OSE, 2) the carcinomas in the ovary were positive for PAX8, a known marker of müllerian tissues that is negative in OSE (29), and 3) despite extensive serial sectioning, we did not find continuity between the ovarian carcinoma and the OSE.

There are several differences between the model presented here and previously reported mouse ovarian cancer models. The majority of previous models relied on OSE transformation and yielded either undifferentiated or poorly differentiated carcinomas(9, 30, 31), endometrioid carcinomas(30, 32), cystic adenocarcinomas (10) and leiomyosarcomas (32-35) but not HGSC as seen in humans and the mogp-Tag mouse. While these mice have taught us a great deal about OSE transformation, the findings are not surprising considering that the OSE is mesothelial in nature and not müllerian as the majority of EOC appear. The mogp-TAg mouse is not the only model that targets FT epithelium. Kim et al (36) reported a conditional knock out of Dicer and Pten in tubal epithelium that was shown to produce carcinomas and metastasis. However, the primary tumors were reported to arise from the FT stroma not the epithelium, which is not believe to be the case in human HGSC. Interestingly, another model, which consisted of inactivating p53 and Rb in mouse OSE through injection of a CRE-adenovirus in the bursa of animals with floxed copies for these genes, yielded both differentiated and poorly differentiated epithelial tumors {Flesken-Nikitin, 2003 #1963}. The differentiated tumors exhibited serous differentiation, but it is worth noting that the Cre adenovirus injected in the bursa may infect FT cells as well as the OSE, possibly explaining the serous differentiation of some of the cancers identified. Another feature of the current model is that, in contrast to the previous genetically engineered models, the mogp-TAg mouse exhibits easily identifiable precursor lesions. The presence of both of these precursors in the FT is particularly important because it can potentially shed light on the early events in high-grade serous tumorigenesis.

The *Ovgp1* promoter used for TAg expression was specific as we did not observe cancer outside the genital tract in females. However, we did observe sarcomas in the uterus of older mice, which is consistent with the observation that the stroma also expresses the TAg. In order to eliminate this complication, hysterectomy could be performed prophylactically in mogp-TAg females at an early age and we are currently exploring this possibility. In any case, while the sarcoma is certainly an unwanted byproduct of *Ovgp1*-mediated TAg expression, we do not believe that it affects the epithelial phenotype seen in the model.

Examination of the uterus, which was enlarged and inflamed in all the female mogp-TAg mice, revealed non-invasive malignant lesions reminiscent of endometrial intraepithelial carcinoma (EIC)(data not shown), the precursor of uterine serous carcinoma (USC) in humans (37). However, we failed to identify invasive carcinoma in the uterus of any of the mice. It will be interesting to examine additional mice and see whether USC can be identified in our model. Nonetheless, it is intriguing that mogp-mice also develop EIC, demonstrating that this model can recapitulate many of the lesions associated with human pelvic serous cancer.

In order to further characterize the lesions found in these mice we performed gene expression analysis of FTE obtained from mogp or WT mice. The expression patterns were clearly different between normal and diseased tissue (Fig. 4), which allowed the identification of multiple genes and pathways abnormally regulated in the mogp FTE. In particular, the p53 pathway was prominently deregulated with multiple downstream genes abnormally expressed. Topoisomerase II alpha, a gene previously shown to be regulated by p53(38) and implicated in breast cancer development (22, 39) was shown to be elevated in mSTICs, the early HGSC lesions identified in our mouse model. TP53 and Topoisomerase II alpha were concurrently expressed in the lesions, a finding consistent with previous observations made in advanced ovarian cancer samples (40). Most interestingly, Topoisomerase II alpha was also elevated in human STICs and ovarian cancers, not only validating our mouse model as relevant to human ovarian cancer, but also identifying a new biomarker. Topoisomerase II alpha is a target for anthracyclines, such as doxycycline, a drug used in platinum-refractory ovarian cancer. Expression of Topoisomerase II alpha may therefore have implications in the treatment of this disease. Interestingly, Topoisomerase II alpha expression has been associated with poor survival and platinum resistance in ovarian cancer {Kucukgoz Gulec, 2012 #2247}.

In summary, we report a new ovarian cancer model based on transformation of FT epithelium. The model exhibits features strongly reminiscent of STICs in humans and, importantly, in some cases also display cancer implants in the ovary. The model may therefore represent a useful tool for the study of ovarian cancer progression. In addition, since the model recapitulates the earliest events in serous ovarian cancer development, it may be useful in the identification of novel strategies for prevention as well as for treatment of this disease.

Table 1. Top 20 up regulated genes in mOp-Tag mouse FT relative to Wildtype FT - Illumina Bead Array Results

Gene Name	Gene Symbol	Z ratio	Fold Change
proline-rich acidic protein 1	Prap1	13.38	38.26
aldo-keto reductase family 1, member B7	Akr1b7	10.37	17.53
protein regulator of cytokinesis 1	Prc1	10.53	14.48
histone cluster 1, H2ad	Hist1h2ad	9.69	11.50
histone cluster 1, H2ah	Hist1h2ah	9.51	11.29
PDZ binding kinase	Pbk	9.31	11.03
topoisomerase (DNA) II alpha	Top2a	8.98	10.37
histone cluster 1, H2ak	Hist1h2ak	9.57	9.57
minichromosome maintenance deficient 5	Mcm5	8.76	9.41
centromere protein A	Cenpa	8.42	8.75
cyclin B1	Ccnb1	8.18	8.59
cadherin 16	Cdh16	8.05	8.53
histone cluster 1, H2af	Hist1h2af	8.55	8.41

nucleolar and spindle associated protein 1	Nusap1	8.21	8.41
cyclin-dependent kinase 1	Cdc2a	7.88	8.26
histone cluster 1, H2ag	Hist1h2ag	8.12	8.21
kinesin family member 23	Kif23	7.96	8.06
histone cluster 1, H2ao	Hist1h2ag	8.26	8.04
cell division cycle associated 3	Cdca3	8.07	7.96
baculoviral IAP repeat-containing 5	Birc5	7.87	7.78

Note: Shaded cells highlight those genes known to be regulated by TP53

Figures and Legends

Figure 1

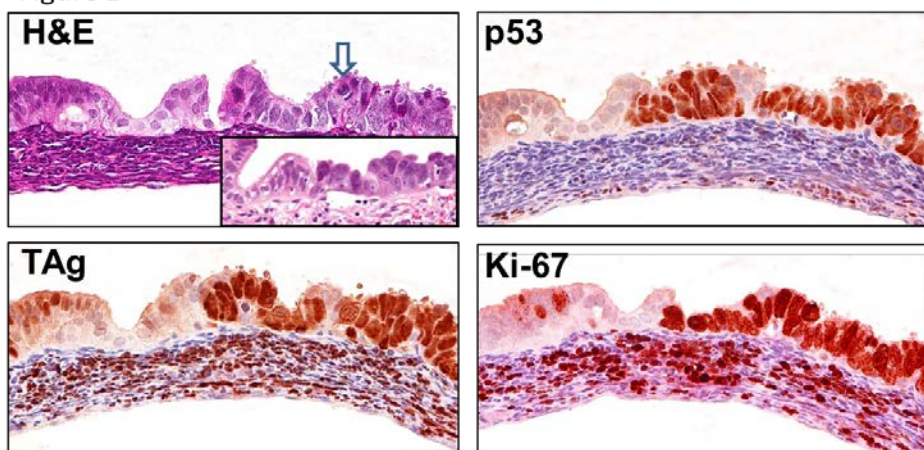


Figure 1. mogp-TAG FT contains lesions (mSTICs) similar to human STICs. H&E staining of a typical 7-8 week old mogp-TAG FT. The blue arrow points to the presence of a lesion, mSTIC, with abnormal chromatin, epithelial stratification, apoptotic bodies and nuclear enlargement, all characteristics of human STICs (human STIC shown in inset for comparison). Adjacent serial sections with immunohistochemical staining against TAg, p53, and Ki-67. All three markers exhibit intense and diffuse positive staining in the mSTIC area of the FT epithelium. Ki-67 and TAg are positive in the stromal area as well.

Figure 2

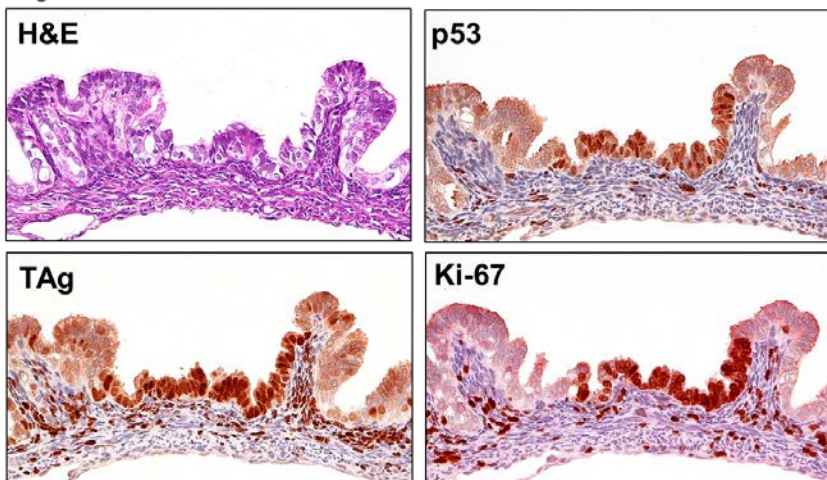


Figure 2. Mouse p53 signature. H&E staining of a typical 6 week old female mOGP-Tag mouse shows an area of the epithelium which appears morphologically normal. Immunohistochemical staining with p53 shows strongly positive and diffuse staining of a portion of the normal FT epithelium: the mouse p53 signature. The

area also exhibits staining for TAg in the p53 signature. Ki-67 staining in the mouse p53 signature indicates that the lesion is proliferative.

Figure 3

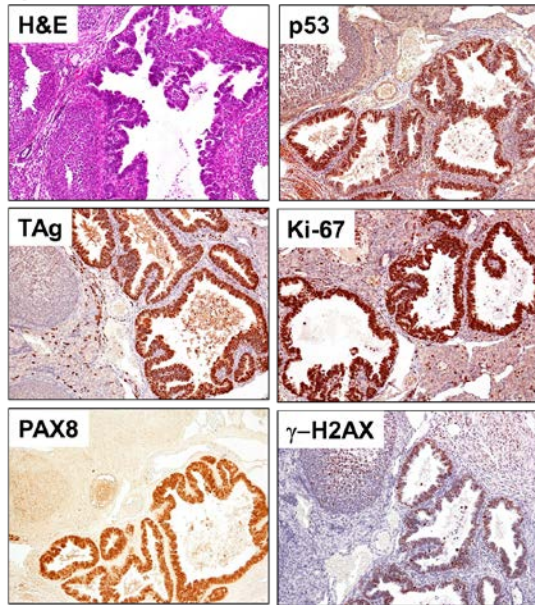


Figure 3. Invasive serous carcinoma of the ovary. H&E of the ovary a 10 week old mouse shows the pathological features of the invasive serous carcinoma that include papillary structures and condensed chromatin. TAg IHC is strongly positive in the stratified epithelium of the invasive carcinoma. The carcinogenic area also demonstrates strong staining for p53, Ki-67, the müllerian marker PAX8 and g-H2AX. 56% of the mice examined have similar findings in their ovaries.

Figure 4

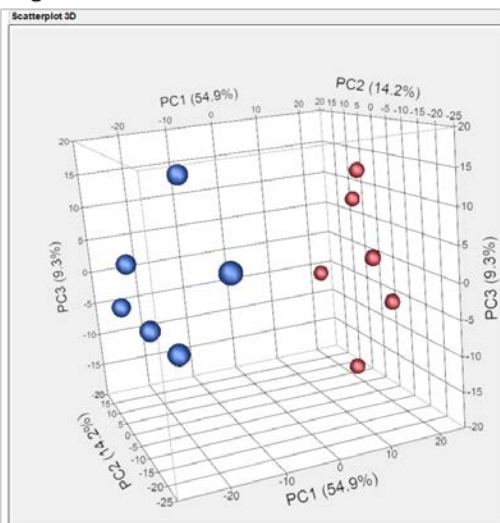


Figure 4. PCA analysis of gene expression in mOgp-Tag and WT mice. FT from mogp-Tag mice (represented by blue dots) showed a distinct pattern of gene expression compared to FT form wt mice (red dots) as depicted both by the PCI values and the visual appearance of the 3D scatterplot.

Figure 5

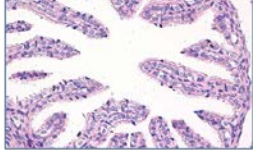
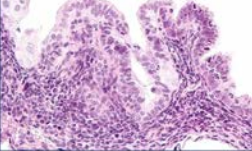
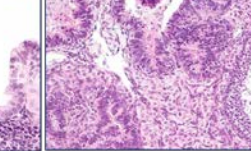
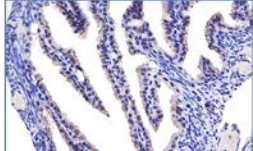
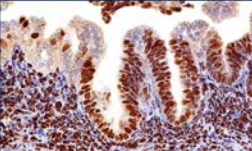
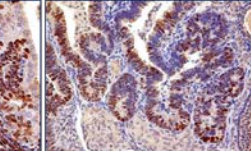
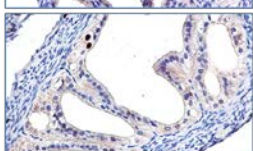
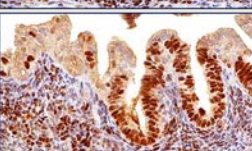
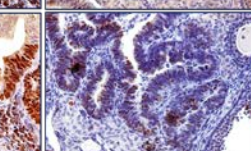
WT FTE	mOGP-Tag FTE	mOGP-Tag OV inv
		
		
		

Figure 5. TP53 (tumor protein p53) and Top2a (Topoisomerase II alpha) are concurrently expressed in mOgp-Tag mouse lesions. WT C57/BL FT epithelium, mOgp-Tag FT epithelium (FTE) and metastatic mOgp-Tag ovary were stained with H&E (top row), P53 antibody (Middle row), or Topo2 antibody (bottom row). These serial sections of representative organs show concurrent IHC staining for both p53 and Top2a.

Figure 6. Human Topoisomerase II alpha IHC. High-grade serous carcinoma (A, C) and Human FT STICs (STICs) display strong diffuse Topoisomerase II alpha staining. Low-grade serous carcinoma (B, D) exhibits sparse staining and normal human FT epithelium (NFT) is negative. Blue arrow indicates the transition site from normal epithelium to a STIC. A, C: H&E staining; B, D, E: TOP2A immunostaining. F. Expression levels of Topo2 were evaluated in normal human FT epithelium samples (NFT), Low Grade Serous Carcinoma samples (LGSC), High Grade Serous Carcinoma samples (HGSC), and Serous Tubal Intraepithelial Carcinoma samples (STIC). The most significant differences are found between NFT and carcinomas: LGSC, HGCD and STICs. There is also a significant difference between LGSC and both STICs and HGSC. There is no significant difference between STIC and HGSC.

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Project 5 progress

Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents, as OCPs in particular are known to prevent ovarian cancer and impact survival. Research site: Johns Hopkins University

PI: Kala Visvanathan, MD, MHS

Collaborating Sites: University of Toronto (Steven Narod, MD and Patricia Shaw, MD: Co-investigators), Memorial Sloan Kettering Cancer Center (Douglas Levine, MD and Robert Soslow, MD: Co-investigator), Yale University (Harvey Risch, MD, PhD, Vinita Parkash, MD and Ellen Matloff, MSc, MS, CGC: Co-Investigators)

Section II. Progress to Date

Since the last progress report we came across some insurmountable hurdles that made us rethink our approach to Aim 1 and introduce a pilot study within the aim to deal with some of these issues. This new strategy will also inform our tissue selection in Aims 2 and 3.

In Aim 1 we originally proposed to determine the prevalence and location of STICs by retrospectively collecting an extensive amount of tissue from the ovaries and fallopian tube of over 500 high-risk women. However in the implementation of our plan despite getting in put from numerous pathologists and clinicians we came across a number of real life challenges. 1. In clinical practice there is marked variation in number of tissue blocks generated for each fallopian tube and ovary specimen. This practice is not standardized which we may eventually recommend. As a result if you are sampling a set number of tissues per block as we proposed this could add hundreds of additional slides to the review of any one case. This was determined to not be feasible in terms of pathological review of these slides and cost. We also did not have a scientific way in which to reduce the number of slides sampled.. This problem only became apparent as we looked at large numbers across the sites. 2. The evolving rules regarding the amount and sampling of tissue at each institution has come under greater scrutiny in the last few years as more tissue work is being done. This means that there are limitations to what we can get and this varies across institutions. We did check at the time of the grant. 3. The increasing costs in obtaining tissue, cutting and transporting this tissue as well as reviewing and staining the tissue. On a positive note a review of the literature did not find any publication that was able to generate robust estimates on the prevalence of STICs they all had limitations. We strongly believe that determining the prevalence of STICs in high-risk women is critical both for the design of future clinical studies as well as interpreting results in the research setting. This information is also needed to determine a good sampling strategy for STICs in the clinical setting using minimal tissue (this will be testing in part 2 of Aim 1). There is currently no standard protocol.

Therefore in consultation with all the pathologists on the study and the biostatistics core we decided to add an important sub aim.. (The amendment has just been approved by IRB and will be forwarded to DOD). We will conduct a pilot study among a subset of the population eligible for Aim 1) N = 95). In these women we will conduct extensive sampling and IHC staining (P53 and Ki67) of their ovaries and fallopian tubes (i.e. sampling every tumor block and the top and bottom of all blocks). Central pathologists will review all slides to determine the prevalence of STICs. The number 95 was based both on a series of power calculations and what was feasible across the sites . Sequential cases from each institution will be obtained (to minimize any selection bias) over a 1- 3-year period. Although ideally we would like to sample the entire tumor block this is not feasible but we will be able to sample both the bottom and top that has not been previously done. We will use the results of this study to generate a more realistic sampling strategy and the minimum number of slides that needs to be sampled. We will use our sampling strategy in the larger study of (> 500 cases). We will also potential validate our findings in a subset of the

larger group of samples. The cases for this pilot study have already been identified at MSKCC and Hopkins and are currently being identified at Yale. Toronto also identified cases but their pathology department will not allow them to flip the block as it requires embedding the tissue in paraffin. We will use additional cases at other sites. They will contribute to the larger study; the majority of cases used in the pilot study will also be used in aims 2 and 3.

We also continue to build the pathological and epidemiological databases for project 5. Over 500 + cases have been identified and epidemiological data from >400 has been inputted. (over 100 + variables) along with path reports from 3 sites. This is from the retrospective study. Identification of cases is still ongoing while data cleaning of this data has started. We are now in a position also to start prospective collection. We hope to complete cleaning and acquiring all the data for the retrospective part in the next 3- 6 months. Once the pilot is over we can then immediately start on the other studies. We are also just starting the prospective part we wanted to wait till we had the approach for the pilot clear. All this upfront work will help complete Aims 2 and 3 also.

Although these large epidemiological studies take longer to put together once they are we will be able to move quickly to answer many questions. We expect to publish the pilot in the next 6-8 months and also begin on marker studies (Aims 2 and 3) with publications quickly following.

Task 1. Obtain approval for the addition of questions for prospective collection from all site IRBs (Aim 1) and also for the transfer of existing epidemiological and clinical data to JHH de-identified for the retrospective study (Aim 1 and 2). Obtain approval from USAMRAA for human subject's research for entire protocol (Months 1-6).

Progress: Approvals have been obtained for all the retrospective and are in process for the prospective collection. We will move forward on this now as waiting to have pilot in place.

Task 2. Identification of study population for retrospective study based on eligibility criteria from all sites (Months 1-6).

Progress: There has been substantial progress on this aim. Epidemiological data and pathology reports on over 483 cases have been identified. Data entry is still ongoing. Ongoing for approximately 100 at this stage. We have not sought cases from Yale yet due to budgetary constraints but hope to obtain some additional funding (which has been submitted for). The path reports of these cases have also been uploaded. This has required close collaboration between the epidemiologist/clinician and pathologist at each site. Data cleaning is about to begin of the master epidemiological data at Johns Hopkins.

Task 3. Comprehensive pathology review of samples. A comprehensive master dataset that includes epidemiological and pathological data from approximately 550 women to be used for cross-sectional and case-control study designs will be created (Months 6-18).

Progress: This is being done as we continue to collect additional samples (described above) we are also in the data-cleaning phase. This resource may become larger than initially proposed but with less extensive sampling. The part that has not happened is the movement of any tissue yet as that will depend on the results of the new pilot study (Aim 1 a) described above. Dr. Visvanathan and her staff have overseen the development of the path database from the beginning.

Task 4. Analyses for Aims 1a and b will be completed to determine the prevalence, location and frequency of the specific lesions in the FTEs (STICs, STILs, and p53 signatures) and OSE, as well as CICs in the ovaries, overall and by BRCA mutation status. Correlations between each of the different types of lesions will be determined. Manuscript preparation Aims 1a and b (Months 6-18).

Progress: This Aim has been modified as described above to include a third aim and that is a pilot study first to determine the prevalence of STICs after extensive sampling of tissue and to work out the best tissue collection strategy for the larger epidemiological study (as described above). We are confident that the pilot study will be completed in 6-8 months and published and we will be able to move quickly on the larger study.

Task 5. Analyses for Aim 1c the association between exposures (both risk and protective factors for ovarian cancer) and each type of prevalent lesion will be examined through cross-sectional studies. Manuscript preparation for Aim 1c (Months 12-24).

Progress: Pending. Infrastructure is being put in place already.

Task 6. As described above, merging of epidemiological and pathological data for aims 1 and 2 will be performed and a comprehensive master dataset to be used from which matched case-control studies will be identified for Aim 2 (Months 6-18).

Progress is described above.

Task 7. Case-control sets for molecular analyses of the FTE and ovary samples from Aim 1 will be used to assess a panel of markers. Cases will be defined by the lesion/region of interest (i.e. STICs, STILs, p53 signatures, CICs, and/or morphological changes in OSE). Controls will vary depending on the analysis: marker expression within a specific lesion will be compared to (1) adjacent normal tissue from the same case and (2) normal tissue from women with no identifiable lesion. For the latter comparison, 2 controls will be matched to each case from the same research site, within +/- 2 years of age at the time of surgery. To conserve this valuable tissue, and maximize efficiency, the same controls will be reused for subsequent case-control analyses where possible (Months 18-24).

Progress: Pending.

Task 8. Molecular analyses to determine molecular profile of each lesion type will be performed on case control sets. The resulting laboratory results will be merged with existing epidemiological data and analyses will be performed (Months 24-48).

Progress: Pending.

Task 9. Statistical analyses of case-control sets will be completed for each lesion type and panel of markers. Multiple manuscripts will be generated from Aim 2 based on the different lesions and also markers. These will be prepared and submitted (Months 24-48).

Progress: Pending.

Task 10. High-risk women considering BSO in the next 2 years, and meeting the same eligibility criteria used in Aims 1 and 2, will be prospectively enrolled at each site. Information on NSAIDs, OCP and Vitamin E use as well as other ovarian cancer risk/protective factors will be collected through questionnaires completed within 2 years of surgery (N ~ 300-400) (Months 6-48).

Progress: We have designed a prospective questionnaire to be implemented at Sloan Kettering once significant progress has been made on the retrospective study and have created additional questions on medication use to be added to the questionnaire at Toronto. This is being reviewed we delayed implementation to get the pilot project going but will now move forward.

Task 11. Ongoing comprehensive pathological review and merging of epidemiological and pathological data will occur (Months 6-50).

Progress: Pending as relates to prospective data collection.

Task 12. Complete merging and data management of Master dataset (Months 48-50).

Progress: Pending. This is for prospective data

Task 13. Analysis of prospective data with respect to NSAIDs and OCP use will be completed: a) Associations between OCP/NSAID/Vitamin E use and prevalence of lesions will be evaluated, overall and stratified by BRCA mutation status as in Aim 1, and b) associations between use of these substances and molecular markers identified in Aim 2. Manuscripts for Aim 3 will be prepared and submitted. This aim will be informed from data generated in Aims 1 and 2 (Months 48-60).
Progress: Pending.

Section III. Problem Areas of Project 5

The major obstacle has been what I described in detail above and that is the need to modify our strategy for Aim1 by the addition of a sub aim. At the same time we have continued to move forward on all other aspects of the projects so that once we work out a sampling strategy based on the pilot we can move quickly to implement.

Section IV. Future Work in Project 5

In the next 6 -8months –we hope to complete the pilot study in 95 cases. This will be the first study to characterize in detail the location and the prevalence of STICs. It will also help us determine a less intensive but feasible sampling strategy for sampling the tissue from the 500-700 women that are part of project 5. In addition it will inform clinical practice, as there is currently no standardized protocol on how to best sample for STICs. Our goal is to have the pilot study published within next 8 months.

In the next 6 months we will prepare the master epidemiological data by cleaning all the > 271 variables) and review the path reports etc. and begin to design the studies for Aim 1 and 2 as well. We will continue to input epidemiological data on potential cases and controls as well as pathological records both from retrospective and prospective collections

Administration (Admin) Core, Biostatistics/Bioinformatics/Epidemiology (BBE) core, and Pathology (Path) Core

Since the integration of the three cores has been integrated to each other, we would like to combine their progress in this section to avoid repetition. Because the tasks related to the three cores are relatively generic and are applicable for the entire research period, therefore, we will rather report the specific progress related to the cores.

Section II. Progress to Date:

The Ovarian Cancer Symposium of the DoD consortium meeting was held at the Memorial Sloan Kettering Cancer Center in May 2014. Thanks to the facility at MSKCC and the excellent organization from Administration core and the hosts, Dr. Levine and Dr. Soslow (the co-investigators), the conference was highly successful. In addition to all the key personnel and investigators from this consortium project and OCRP, there were at least 150 people who attended this symposium. Young investigators from different sites had also the opportunity to present their research work related to the consortium projects and meet with other colleagues and senior investigators in the ovarian cancer research field. There are several group meetings during the symposium that the PIs from each project discuss the potential problems and future directions with advisory board members. New collaborations had been created and exciting research that stem from the current consortium projects was discussed. For example, Dr. Shih (PI in Project 1) has established new collaboration with Dr. Ronny Drapkin (from Harvard) to look at the molecular changes involving early lesions in ovarian cancer development. Dr. Drapkin's new mouse model has been transferred to JHU for new studies in Project 4. The Administration Core together with the Pathology Core and several PIs at JHU have tried their best effort to recover from the serious flood at CRB2 on October 30th, 2012 due to Hurricane Sandy. The reagents, mice, and other supplied have been restored to the pre-damaged status. In collaboration with Administration Core, the Pathology Core is up and running. The progress of this core is summarized below. PathCore at JHU met twice a month (1st and most 3rd Tuesdays). These meetings covered follow up of day to day operations and issues related to consortium projects and Pathology Core, especially the tissue collection and compliance. During these meetings conference calls also were made to update the study sites (1/22/1013 Dr Shaw). Slide collection has started for PIs (please see Table below) Pathology Core organized barcoded label creation and shipment. Also

		JHU	MSKCC	YALE	TORONTO
Demographics		15 CASES	15 CASES	N/A	25 CASES
Clinical Information		15 CASES	15 CASES	9 CASES	25 CASES
Slides	H&E	15 CASES	15 CASES	9 CASES	N/A
	p53	IN PROCESS	15 CASES	9 CASES	N/A
	Ki67	IN PROCESS	15 CASES	9 CASES	N/A
	LamC1	IN PROCESS	IN PROCESS	IN PROCESS	N/A
Frozen Tissue Collection		N/A	N/A	N/A	N/A
Pathology reports upload		15 CASES	15 CASES		25 CASES

provided all sites with slide boxes. Currently all sites are continuing to identify new cases, getting tissues cut per SOP for identified cases and de-identifying pathology reports before uploading the to the Database. Pathology Core organized Teleconference trainings for Ovarian Biorepository Database (MSKCC, Yale, Toronto) and continues to provide all the necessary support. Ovarian Database is regularly updated with

new cases and fine tuning. Moreover, conception of SOP for pilot project to minimize slide cutting expense for project 5 has been discussed. The Biostatistics component of the BBE Core has provided all the necessary support to the consortium investigators who need the biostatistical assistance in study design and data analysis.

Section III. Problem Areas for the Cores

We do not expect major problems for the three cores.

Section IV. Future Works in Cores

The future works for the three cores correspond to what have been proposed in this consortium study. Briefly, the Admin Core will continue overseeing and manage performance of the Path and BBE Cores and the

Research Sites. Specifically, the Admin Core will promote the integration and coordination of research activities, ensure new IRB protocols and additional biosafety regulations are in place, coordinate intellectual and material property, organize regular meetings of the investigators including the Annual Ovarian Cancer Symposium, monitor project performance, prepare annual progress reports, organize and make travel arrangements for investigators and ensure dissemination of data and publication of major findings as well as maintaining the ovarian cancer web site. The Path Core will be responsible for tissue repository and the BBE core will ensure that data are consistently collected and entered into the Consortium database. As described in the task, the Path Core will collect tissue and analyze it histologically according to standardized practices and the algorithm that we developed for this program, perform tissue-based assays, and annotate this material in a centralized fashion while assuring quality. The Path Core will also oversee functions related to storage and transportation of tissue samples in order to supply researchers with pathologically characterized material. Both Path and Admin Cores will work together to coordinate the shipment of slides among Research Sites and the Pathology Core. The BBE Core will provide biostatistics, bioinformatics, and epidemiology consultation and support to all members of the Consortium. Core members will assist in the design, collection, storage, visualization, analysis, quantitative modeling, and interpretation of the data arising in the course of Consortium activities. Centralizing biostatistical and bioinformatics support within the Core will ensure that the necessary expertise will be available for all projects, facilitating the coordination and integration of data throughout the Consortium in a consistent fashion.

Section III- Problem Areas

The problems related to individual projects have been described in each project section.

Section IV- Work to be performed during the next reporting period

The future works related to individual projects have been described in each project section.

Section V- Administrative Comments (Optional)

Section VI- Meetings

We will schedule the next DoD Consortium PI meeting in conjunction with the Ovarian Cancer Symposium in May 2014. The location has not been determined but probably will be at Toronto, Canada. EAB and RCA meetings will also be scheduled at that time.